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CONTENTS

	PAGE
The Swelling of the Arm After Operations for Cancer of the Breast—*Elephantiasis Chirurgica*—Its Cause and Prevention. (Illustrated.) By William Stewart Halsted	309
Studies on Blood. The Vitally Stainable Granules as a Specific Criterion for Erythroblasts and the Differentiation of the Three Strains of the White Blood-Cells as Seen in the Living Chick's Yolk-Sac. By Florence R. Sabin	314
Some Oxidation Mechanisms of the Cell. By F. Gowland Hopkins	321
Studies on Some of the Non-Lipoid Components of Blood Serum in Relation to Its Antihemolytic Property. By Hallie M. Clark, Rhea H. Zinck and Frank A. Evans	328
The Nucleus in the Human Restiform Body. (Illustrated.) By K. Hirose	336
Books Received	338
Notes on New Books	340

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THE SWELLING OF THE ARM AFTER OPERATIONS FOR CANCER OF THE BREAST—*ELEPHANTIASIS CHIRURGICA*—ITS CAUSE AND PREVENTION

By William Stewart Halsted

Nearly 40 years ago, probably in 1882, I had occasion to excise thoroughly the lymphatic glands from both groins of a young man, and there resulted a swelling of the scrotum greater than I have ever seen as a consequence of this operation. Much perturbed, I consulted Dr. Charles E. McBurney who at this time was interested in the management of similar cases, and together, for a year or more, we attempted with inconsiderable success to reduce, chiefly by strapping with adhesive plaster, the distressing dimensions of the swelling. Two and a half years ago this patient, to whose misfortune I had repeatedly referred in my clinics at The Johns Hopkins Hospital and whom I had not seen for about 38 years, came to Baltimore to consult me in regard to another matter. The scrotum, although much reduced in size, was conspicuously large and incommodious. Especially interesting was the account which he gave, in response to careful questioning, of recurrent attacks of redness and increased swelling accompanied by digestive and constitutional disturbances. These attacks, not infrequently ushered in by chills and fever, were milder and less frequent than formerly but still occurred at least once or twice in the course of a year.

Œdema following operative blocking of the lymphatics is most frequently observed after the radical operation for cancer of the breast. For many years I was unable to account for the fact that in some instances a year or two or more after this operation and without return of the disease there would occur, suddenly or perhaps slowly, a swelling, occasionally very great, of the upper extremity. Consultations on the subject with surgeons in this country and abroad were not very helpful, the opinion frequently vouchsafed being that swelling due to obstruction of lymphatics was hard and appeared late, whereas the swelling which followed blocking of the veins was relatively soft and supervened promptly. In the spring of 1914 the Director of one of the greatest clinics in Germany expressed to me the opinion that the typical operations upon the axilla in cases of carcinoma of the breast were unnecessarily radical, and that in order to forestall the excessive swelling of the arm which followed in such a large percentage of the patients he was stripping out the axillary contents in a manner much less thorough. Since adopting this modification he was confident that swollen arms were less frequent in his clinic and that the percentage of recurrence had not been increased thereby.

For many years I have entertained the view that although blocking of the lymphatics and occasionally also of the veins was the underlying factor, infection played a conspicuous part

in the determination of the amount of the swelling and the time of its manifestation. The facts observed in the following case sustain this opinion most convincingly:

A woman (Surg. No. 41383) on whom I had performed at The Johns Hopkins Hospital our radical operation for mammary carcinoma on November 18, 1916, consulted me on May 24, 1920, in regard to a swelling of her arm which had appeared suddenly (within three or four days) three years and three months after the removal of the breast. There had been no swelling whatever of the arm at any time subsequent to the operation prior to an "attack" in February, 1920, in the course of her convalescence from influenza. This attack, as she terms it, was ushered in by nausea, a chill and high fever. The arm promptly began to swell and there appeared "redness in streaks" from the shoulder to the wrist; in a few days the redness was diffuse and the swelling of the arm had become distressingly great; the hand resembled a "boxing glove," and pressure on it with the fingers produced "deep pits." In eight or ten days the redness had vanished and the swelling was rapidly decreasing. During the three months prior to this her second admission to The Johns Hopkins Hospital there had been a less rapid reduction in the size of the affected (left) limb, which about the middle of the arm measured in circumference 9.5 cm. more than the other, and at the middle of the forearm, 3.5 cm. more. No glands were palpable above the clavicle, and there seemed to be no abnormal fullness or resistance below it. In the skin at the outer-upper edge of the grafted area there was recurrence of the carcinoma—two nodules, not ulcerated, one about the size of a filbert, the other smaller than a split pea.

The immediate cause of the swelling was undoubtedly the local infection, for during the three years and three months prior to this there had been nothing to indicate a blocking of the lymph channels.

Eight days after the second admission (June 1, 1920), the patient was operated upon by Dr. Mont Reid, our resident surgeon. The second and a part of the third portion of the axillary vein were found to be completely occluded by the new growth, which was continuous with the larger of the two cancerous nodules in the skin. The entire axillary vein and the recurrent growth were excised in one piece, the disease being given a wide berth. On examination of the specimen it seemed quite clear that the vein had been invaded from without, and had probably been occluded long before the attack of infection. After this operation the swelling rapidly subsided and in the course of two months the arm had almost regained its normal dimensions.

In this case, as in a number of others observed in our clinic, the occlusion of the axillary vein plus the excision of the axillary lymphatics had not been followed by any swelling of the arm.

A few months ago I interviewed a patient (Surg. No. 17659) who each year for the sixteen years following an operation by me at The Johns Hopkins Hospital for cancer of the breast has had one or two and occasionally three or four attacks of redness and swelling of the arm on the operated side. The first symptoms, malaise and nausea, were quickly followed in the severe attacks by a chill and fever and then by slight redness and increased swelling of the arm. The arm of this patient was observed to be swollen a few days after the operation, the swelling being attributed in part to the excision of the axillary lymphatics, but in great measure to a slight infection of the wound along the incision onto the arm.

At that early period, and indeed until perhaps after the introduction of our present operation, about eleven years ago, we believed that, quite independent of infection, swelling of the arm might be expected after the radical operation for mammary cancer.

The initial account of the operation for cancer of the breast which bears my name lies buried in the second volume of the Reports of The Johns Hopkins Hospital (1891) under the title "The Treatment of Wounds with Especial Reference to the Value of the Blood Clot in the Management of Dead Spaces,"[1] and is known only to a few.

Nine years have now elapsed since the publication of my paper on "Developments in the Skin-Grafting Operation for Cancer of the Breast,"[2] in which a modified incision and radical changes in the manner of closing the wound were advocated; but I was not then able to offer an explanation altogether satisfactory to myself of the fact that the swelling of the arm which not infrequently followed our original procedure became almost immediately after the adoption of the modifications described in the latter paper a complication of rare occurrence.

Our operation of to-day is the one which with few exceptions has been employed at The Johns Hopkins Hospital for the past eleven years. Prior to its adoption we had not infrequently observed that extreme abduction of the arm was prevented by a cicatricial, cuticular band in line with the scar of the incision onto the arm. In no case, however, did the patient consider herself sufficiently incommoded by the restriction of movement in this direction to accede to the proposition that the tugging band be divided and the defect covered by skin grafting.

The problem thus thrust upon us was readily solved: the incision down the arm, made shorter and shorter, was soon abandoned altogether, and the skin-edge at the upper margin of the wound was tacked with fine silk sutures to the first intercostal muscle and its fascia in such manner as to raise the axillary fornix to the highest desirable point (*vid.* Fig. 1). Thus the possibility of tug on any part of the skin or scar was eliminated.

No effort was made to approximate the cut edges of the skin at the upper third or perhaps upper half of the denuded area; on the contrary, the flaps, if so they may be called, were pressed away from the wound's centre—the outer flap upwards and outwards, the inner flap upwards and inwards—and stitched to the underlying muscles of the thoracic wall. Thus there was secured for the infraclavicular and axillary regions a superabundance of skin, and entire freedom of arm movements in all directions was assured. Furthermore, by the tacking of the margin of the flap close up to and sometimes even under the subclavian vessels (*vid.* Figs. 1 and 2 and 4 to 12), there could be no dead space below the clavicle such as results in plastic operations from the attempt to approximate the margins of the flaps—the dead space whose arc, formed by clavicle, first intercostal muscle, and first rib, is subtended by the more or less tightly drawn tegmen. The lower half or more of the denuded area was to a limited extent

[1] W. S. Halsted, The Treatment of Wounds with Especial Reference to the Value of the Blood Clot in the Management of Dead Spaces. The Johns Hopkins Hospital Reports, 1891, II, p. 255.

[2] W. S. Halsted, Developments in the Skin-Grafting Operation for Cancer of the Breast. Jour. Amer. Med. Assn., 1913, LX, p. 416.

covered by gently drawing the free margin of the skin towards the centre of the wound and tacking it to the underlying muscles, although the size of the grafted area is of little practical consequence to the patient, and only slightly more time is consumed in covering with the large grafts a greater than a lesser surface.

More was accomplished by this new method than had been anticipated, for not only was the normal range of arm movements secured but there resulted other benefits unforeseen. Swollen arms of dimensions sufficient to distress or annoy the patient were no longer observed, marginal necroses rarely occurred, and the grafts took throughout with few exceptions.

Now it is within the experience of every surgeon, especially of those who employ plastic methods to cover the defect, that frequently marginal necroses of the flaps occur and occasionally sloughings of considerable dimensions, and that it is usually in these cases that the swelling of the arm is most pronounced. Attendant upon the necrosis there is infection, and inflammatory reaction in varying degree, and the greater the reaction the greater in general the swelling of the arm immediate and ultimate.

Moreover, there are, as I have said, cases in which months or even years after the operation on the breast the arm becomes swollen from a cause other than the recurrence of the carcinoma.

Recurrent carcinoma being excluded, may not the later swelling of the arm have the same cause, whatever that may be, as that which appears soon after the operation? What is the cause? What the explanation of the fact that after the radical operation, the world over, for cancer of the breast the arm so frequently swells and sometimes to such degree that amputation has been proposed and indeed made for relief?

Why is it, furthermore, that a modification in our method of closure of the wound, the operative details otherwise being essentially unchanged, should so strikingly affect the result as regards the swelling of the arm?

It is not from observations made in our clinic alone but from the testimony of surgeons and radiologists elsewhere that we have been led to conclude that swelling of the arm follows the plastic operations in greater proportion and in more pronounced form than is seen in the cases treated by skin grafting. And, moreover, in the skin grafting operations, which with us trace back to 1895, conspicuously swollen arms became almost a thing of the past after the method of pressing back the flaps at the upper part of the wound, and stitching their edges to the underlying intercostal muscles was adopted.

Something must therefore have been superimposed upon the mere clearing out of the axilla in the ordinary as well as in the extraordinary plastic operations, and in the operations at The Johns Hopkins Hospital which antedated our modified procedure, to account for the so frequent swelling of the arm.

In addition to the evidence furnished by our clinical experience we have the testimony of the many who have studied the causes of elephantiasis filariosa that blocking of the lymphatic glands and vessels is not of itself sufficient to account for the œdema, at least in its pronounced forms.

Dr. F. L. Reichert, who with the assistance of Dr. C. Y. Bidgood has made for me in the Hunterian laboratory, during the past winter, experiments bearing on this subject, has thus far been unable to produce œdema in the legs of dogs by the mere severing of veins and lymphatics. In these amputations all the tissues of the thigh were severed except the femoral artery and vein, the main nerve trunks and the bone; then the divided parts were carefully reunited by stitching. For 7 or 8 days there would be slight swelling of the leg below the line of suture. On subsidence of this temporary swelling the femoral vein (the only undivided vein) would be ligated. No demonstrable increase in the size of the leg occurred after this ligation. Thus in one week or less the lymphatico-venous circulation was reestablished through the line of suture—through the scar. We have it in mind to make the ligation also of the artery at various periods after the amputation.[3] Attempts are being made by us to determine the relative susceptibility to infection of the two hind legs—of the operated and the normal one. We conjecture that the leg operated upon may prove to be more susceptible to infection than the other and also that an abnormal amount of swelling may follow a successful inoculation of the obstructed leg.

These experiments call to mind an interesting paragraph by W. G. MacCallum on "Inflammation in Tissues Separated from Connection with the Central Nervous System."[4]

> An attempt was made to study the cause of inflammation in tissues separated from connection with the central nervous system as compared with that in normal tissues. The mere section of the nerves going to an organ or limb is insufficient, for nerve fibrils accompany the blood vessels. To overcome this an extremity was amputated completely and replaced by anastomosing the blood vessels and bringing together muscles and skin. Inflammatory irritants applied symmetrically to the intact, and to the amputated limb of the dog resulted in the production of quite the same phenomena of inflammation on both sides. The reddening due to the dilatation of the blood vessels

[3] Since the above was written Dr. Reichert has informed me that he has already carried out the suggestion to ligate the artery as well as the vein about a week after the amputation made in the described manner. Neither gangrene nor swelling followed the ligation of these vessels. Thus, within a week of the time of the amputation of the thigh the arterial and venous circulations had been so well restored through the line of union of the divided tissues as to make possible the ligation of the femoral artery and vein without causing any appreciable swelling or anemia of the limb. It remains to note the effect of ligation of the artery with unoccluded vein, and also to determine the earliest period after the amputation at which these vessels can safely be occluded. It is to me a surprising fact that in so short a time the arterial, venous and perhaps lymphatic circulations can be reestablished through a scar. We have it in mind to inject the vessels of the animals so treated with shadow-casting materials and thus, if possible, study with the X-ray the fine channels through which the new circulation is carried on. Conceivably an amputated limb might be reinstated as is the case with severed fingers, ears and noses. One must at least make the experiment. These experimental observations tend to strengthen my belief that swelling of the arm is unlikely to be caused by the mere excision of the axillary lymphatics. It would seem to be quite certain that another factor is needed, namely, infection.

[4] Proceedings of the Soc. for Exp. Biol. and Med., 1910-11, p. 180.

was perhaps slightly more intense on the amputated side than in the intact limb. Evidently, the control of the central nervous system is not at all necessary for the development of inflammatory changes.

May the swelling of the arm after the radical operation for cancer of the breast be prevented? The study of our cases operated upon by the newer method has brought out the fact that excision of the axillary and supraclavicular glands plus resection of the subclavian and axillary veins is rarely followed by noticeable swelling of the arm. On the other hand, we do not deny that obstruction of these lymphatic and venous channels might conceivably alone, without infection, suffice occasionally to produce a moderate amount of œdema. But it is assuredly impossible to assert in any case that infection has played no part in the causation of the swollen arm.

It is unnecessary to stress further the importance of discarding operative methods which clearly predispose to infection, quite aside from the fact that these methods—the plastic ones—tend to deter the surgeon from sacrificing a sufficient amount of skin or, if perchance enough skin has been excised, to increase the danger of swelling and of restricting the range of movement of the arm. In the plastic operations, the wide dissections of the skin extending sometimes to the spinal column, over and under the opposite breast and down to the iliac crest, may conceivably sever lymphatics which might aid in the restoration of the lymphatic circulation. The performer of plastic operations presumably asks himself whether skin enough to make possible the use of his favorite method can be preserved; whereas the advocate of skin grafting who understands its possibilities may sacrifice, without concern about the closure of the wound, as much skin as seems to him desirable. It is unfortunate that surgeons should permit themselves to be placed in the predicament of choosing either to incur greater danger of recurrence or to imperil the success of a plastic method.

During the past year we have been making a careful search for cases of swollen arm following operation at The Johns Hopkins Hospital for cancer of the breast and, although at this writing we are not prepared to submit figures, may state that the records support our view that infection is very frequently, if not indeed usually, the overlying cause of the swelling of arms whose main lymphatic channels have been more or less blocked by operation. The infection may quite conceivably be so mild in degree as to escape the observation even of those intently on the lookout for it.

If the view expressed in this paper as to the cause of the swelling of the arm following operations upon the axilla should prove to be correct the term *surgical elephantiasis (elephantiasis chirurgica)* might be an appropriate one.

The most common cause of the late postoperative swelling is, of course, the recurrence of the disease, a recurrence which blocks new channels. But swelling in its most aggravated form is seen with the recurrences accompanied by inflammation—it may be only the reactive inflammation incident to the rapidly growing neoplasm. Now the question naturally presents itself as to whether the reactive inflammation in some of these recurrent cases may not be partly of bacterial origin—an inflammation superimposed upon the tissues engorged from lymphatic and frequently also from venous obstruction. It would seem to me to have been determined from clinical observation that lymphatic obstruction predisposes to streptococcal inflammation, but it remains for us to confirm this hypothesis by experimental demonstration on animals. We have been impressed with the fact that lymphangiomata seem predisposed to infection, and to recurrent attacks of inflammation.

The clinical and experimental evidence bearing on the part played by streptococcal infection in the production of elephantiasis and elephantoid conditions has been admirably summed up by Matas:[5]

> By elephantiasis we mean a progressive histo-pathologic state or condition which is characterized by a chronic inflammatory fibromatosis or hypertrophy of the hypodermal and dermal connective tissue which is preceded by and associated with lymphatic and venous stasis, and may be caused by any obstruction or mechanical interference with the return flow of the lymphatic and venous currents in the affected parts. In order to bring about the hypertrophy of the connective tissue, which is the distinctive feature of the true elephantiasic state, the mechanical impediment to the lymphatic and venous drainage of the part is not sufficient, because a simple mechanical obstacle, while causing a regional or localized dropsy or lymphedema, will not bring about the characteristic fibromatosis and other histologic changes which are peculiar to elephantiasis. As Unna, Darier and many others have well shown, a simple mechanical edema is incapable of exciting a proliferation of the collagenous connective tissue. We know by clinical observation that edema may exist many years in the extremities and other parts without causing any fibromatosis or hyperplasia of the connective tissue of the parts. Something more than lymph stasis is required, and that something is infection with pathogenic organisms, and especially those of the streptococcal type which find a favorable soil for development in the stagnant lymph stream. The histopathological elements which are essential to complete the picture of elephantiasis are: (1) a mechanical obstruction or blockade of the veins and lymphatics of the region, usually an obliterative thrombo-phlebitis or lymphangitis or adenitis; (2) hyperplasia of the collagenous connective tissue of the hypoderm; (3) gradual disappearance of the elastic fibres of the skin; (4) the existence of a coagulable dropsy or hard lymph edema; and, (5) a chronic reticular lymphangitis caused by secondary and repeated invasion of pathogenic micro-organisms of the streptococcal type.
>
> If we adopt this conception of the histologic process which underlies the pathology of elephantiasis as it is recognized in its endemic tropical types, as well as in the sporadic cases, which may occur in all climates, we can readily appreciate that the histologic process is of a generic character, though it may be initiated by many specific causes—the underlying histologic background remaining, however, always the same. In this manner, we can easily reconcile the many conflicting views relative to the pathology of elephantiasis. The long established duality of classification of the disease into the classic *Elephantiasis tropicum*, which is usually attributed to the presence of *Filaria nocturna*, or the strictly parasitic type of the disease, and the *Elephantiasis nostras streptogenes*, which prevails in all climates, no longer suggests distinct and specific types of the disease. These are histologically alike as morbid processes, and only differ in the primary cause of the lymphatic and venous obstruction which initiates the process. Therefore, while nosologists classify elephantiasis into the

[5] The Surgical Treatment of Elephantiasis and Elephantoid States Dependent Upon Chronic Obstruction of the Lymphatic and Venous Channels. With Case Reports by the Author and Hermann B. Gessner, M. D., School of Medicine, Tulane University of Louisiana, New Orleans. Amer. Jour. of Trop. Dis. and Prev. Med., 1913, I, 60-85.

FIG. 1.—The redundant axillary skin is drawn up by the fingers. The skin has been stitched to the underlying muscles at the margin of the area to be grafted, the apex of which corresponds to the lower border of the first rib. Reproduced with permission of the *Journal of the American Medical Association.*

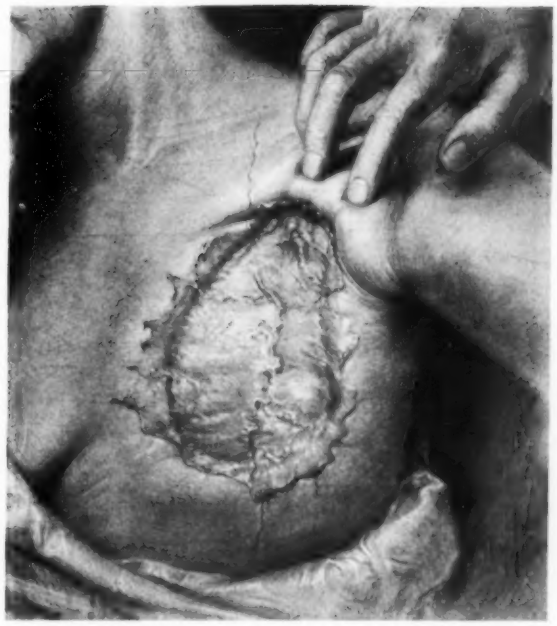

FIG. 2.—The defect has been covered with Ollier-Thiersch grafts. The redundant axillary skin retracted by the fingers, as in the preceding illustration. Reproduced with permission of the *Journal of the American Medical Association.*

FIG. 3.—S. N. 29519. Operation by Dr. Halsted March 30, 1912. Reproduced with permission of the *Journal of the American Medical Association.*

FIG. 4.—S. N. 29519. Three days after operation. The denuded area covered by skin grafts. Silver-foil (high lights) on the skin along the inner and upper margins of the wound. Reproduced with permission of the *Journal of the American Medical Association.*

FIG. 5.—S. N. 51869. Operation by Dr. Reid September 3, 1920. Huge fungating carcinoma; skin metastases; subclavicular, axillary, and regional œdema; swollen arm, forearm and hand. Photograph taken nine months after operation. The swelling of the arm was not increased by the operation. Note the great size of the grafted area and the redundant skin in the axilla.

FIG. 6.—S. N. 49379. Operation by Dr. Reid September 19, 1919. Photograph taken April 1, 1921.

FIG. 7.—S. N. 49808. Operation by Dr. Heuer November 21, 1919. Photograph taken January 18, 1921.

FIG. 8.—S. N. 37632. Operation by Dr. Dandy August 10, 1915. Photograph taken Feb. 27, 1920.

FIG. 9.—S. N. 46656. Operation by Dr. Reid September 27, 1918. Photograph taken February 27, 1920.

FIG. 10.—S. N. 40261. Operation by Dr. Dandy July 12, 1916. Photograph taken March 8, 1918. A graft placed almost at the very top of the axillary fornix.

FIG. 11.—S. N. 42198. Operation by Dr. Heuer February 19, 1917. Photograph taken March 8, 1918.

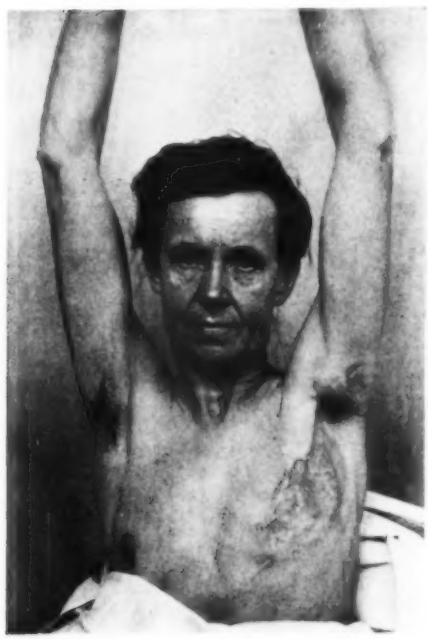

FIG. 12.—S. N. 45095. Operation by Dr. Follis March 1, 1918. Photograph taken February 27, 1920.

previously mentioned groups, it is evident that the elephantiasic process is always one and the same; always maintaining similarity of histologic type regardless of the multitude of causes that may bring it into existence. The one etiologic factor which seems to be inseparable and essential to its pathogeny is infection—frequently repeated—which brings about permanent alterations in the vasculolymphatic apparatus of the skin and its underlying connective tissue. The reason why elephantiasis, as a clinical entity, is so much more frequent in the tropics than elsewhere, is because the conditions which predispose to and favor cutaneous infection as well as lymphatic obstruction, are so much more frequent there than in colder climates; greater exposure of skin areas, especially the lower extremities, to traumatisms of all sorts; greater activity and, consequently, greater irritability of the skin; universal presence of suctorial insects and gross parasites; and greater frequency of eruptive diseases, etc., all of which tend to open wide the portals of infection. It is now pretty well admitted that filariasis is only one of the causes that initiate the elephantiasic process even in the tropics. Certainly, the filariæ are not so frequent a cause of the disease as was formerly believed. The incidence of filariasis is not coincident with that of elephantiasis. For instance, Creighton Wellman (Journal of Trop. Med., 1908, p. 118), while stationed as health officer at the Portuguese Colony of Angola, in West Africa, had many opportunities for investigating the relationship between filarial disease and elephantiasis and in a careful study of fifty cases he never found any evidence of embryo filariæ in the blood, or for that matter, in the blood of five hundred individuals in that colony, whom he examined for evidence of filarial disease. Le Dantec had a similar experience in French Guiana, where elephantiasis is common and filariasis very rare. Prout, in Sierra Leone (1908), observed a great many cases of filariasis in that colony, but met with a similar experience in the Congo, where filariasis is almost a universal disease. Sir Patrick Manson, who immortalized himself by his researches into the mode of propagation of filarial disease through the agency of the mosquito, and was the first to establish the relations of filariasis to elephantiasis, has modified his earlier views on the relationship of filiarial parasitism to elephantiasis. In his work on Tropical Diseases (1907) he now teaches that the mechanical obstruction of the lymphatics caused by *Filaria bancrofti* is not alone sufficient to cause elephantiasis, and that a secondary microbial infection of the obstructed area is necessary to cause the tissue changes of elephantiasis. This same observer writes:

"Lymphatic stasis, by itself, does not cause elephantiasis. The obstruction may cause lymph edema, but not a true hypertrophy of the edematous tissues. If an inflammation (infection) is acquired in a closed area of lymphatic congestion, an event which may result as a consequence of the slightest traumatism, elephantiasis will then develop."

According to Low, if a lymphatic obstruction caused by adult filariæ is followed by an attack of erysipelas, the filarial embryos are killed and their disorganization is a cause of the lymphangitis which culminates in elephantiasis. Clinical observation also abundantly shows that if a lymph scrotum develops as a consequence of filarial obstruction, it will remain a plain lymph scrotum for an indefinite period of time, and will only become an elephantiasic scrotum when repeated attacks of erysipelatous infection follow in the wake of the mechanical stasis and in this way starts the fibromatous process which is the histologic essential of elephantiasis. The burden of this discussion is merely to show that even in tropical climates where filariasis prevails endemically, elephantiasis is a diseased state not subject to one cause, but to many, and, in fact, to any agency that may obstruct the lymph stream provided dermal infection follows in the wake of lymph stasis. We have now come to realize that whatever the cause of the lymph edema may be, the element of infection is the one essential and determining factor in the production of true elephantiasis. In fact, if we accept the views of many writers, such as Le Dantec, Sabouraud and Unna, the progressive fibromatosis which we recognize as *Elephantiasis nostras*, may occur independently of any stagnatory state of the lymphatic or venous circulation, and solely as a result of repeated attacks of a streptococcal infection, which has been regarded by many as identical with the erysipelas coccus of Fehleisen. Moreover, the streptococcal infection of elephantiasis presents all the characteristics of the classical cutaneous erysipelas, with the exception that in elephantiasis it is usually limited to one particular region in the lower extremity, the eruption rarely extending beyond the groin. It is rarely ambulatory or migratory as is the case with the well-defined type of erysipelas.

This association of a streptococcal infection of the erysipelatous type is, we repeat, almost an inseparable and constant feature of elephantiasis, no matter what the original predisposing cause may be. It is also this association of the erysipelatous attacks, with the progressive hyperplasia of the dermal and hypodermal connective tissue of the affected region, that distinguishes true elephantiasis from the elephantoid states. Sabouraud, who has given much attention to the bacteriology of elephantiasis, Unna, Le Dantec, Bockhart, and others, agree that the micro-organism is a distinct *Streptococcus* which cannot be morphologically distinguished from the Fehleisen coccus of erysipelas. Le Dantec describes in addition a special coccus which he designates as the lymphococcus; Renon found the pneumococcus. But there is no question that from the clinical point of view, the recurrent streptococcal infection which is peculiar to elephantiasis is similar to that of erysipelas. In the many cases that have come under our observation, the history of repeated attacks of erysipelas has been invariably connected by the patient with the origin and evolution of the disease. Sir Joseph Fayrer applied the term elephantiasic fever to the febrile manifestations which accompany the appearance of the local erysipelatous rash. The fever is usually ushered in by chill or rigors, and the temperature rises to 103° or 105° F. and is followed by remissions and profuse sweats. The fever usually lasts from two to eight days and defervesces with the subsidence of the erysipelas, which rarely extends beyond the root of the limb. During the attack, the skin of the affected region becomes red, painful, swollen, and looks exactly like the erysipelas rash; the lymph nodes are enlarged and the redness diffuses itself with great rapidity over the entire surface of the limb. After the subsidence of the attack, the skin remains swollen, giving the impression of a soft, doughy edema. The soft consistency gives place to a more permanent hardness, the skin of the affected part never returning to normal. The underlying soft parts unite with the skin, which increases very much in thickness, forming a firm, immovable mass of tissue which continues to grow until it finally assumes a monstrous size (Elephant's foot, Barbadoes leg, etc.) which is characteristic of the disease.

The development of elephantiasis by inoculation of pure erysipelous cultures was experimentally tested by Bockhart, Sabouraud, Le Dantec and others. In accounting for the repeated or recurrent attacks of this streptococcal infection, Unna (Histopathology of the Skin, Walker's translation, p. 493) states:

"It is in the highest degree probable that the true sporadic cases of elephantiasis develop from incompletely healed erysipelas—that is, those which leave behind disturbances in the circulation. The streptococci remain latent in the tissues and in this way excite the chronic proliferative tissue changes which we subsequently recognize as elephantiasis. At first the attacks of erysipelas occur at long intervals, perhaps twice or three times a year, then once a month, rarely oftener. Each time the limb grows larger until in the course of time a distinct elephantiasic state is established. Natural immunity does not appear to be easily acquired; and once the erysipelatous habit, so-called, is acquired, it usually becomes a permanent feature of the disease, though in some cases the attacks become milder and disappear altogether, but not until the elephantiasic state is fully established."

STUDIES ON BLOOD

THE VITALLY STAINABLE GRANULES AS A SPECIFIC CRITERION FOR ERYTHROBLASTS AND THE DIFFERENTIATION OF THE THREE STRAINS OF THE WHITE BLOOD-CELLS AS SEEN IN THE LIVING CHICK'S YOLK-SAC

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In 1920 the writer[37] published an account of a study of the origin of the vascular sytem as it can be made out by watching the living chick blastoderm of the second day of incubation. The method of the origin of vessels can be made out in such specimens, in the area pellucida of the yolk-sac, in stages which range from the time just before the first somite through the stage of about twenty somites.

In the area pellucida there are three well-known layers, the ectoderm, the double layer of the mesoderm lining the extra-embryonal cœlom, and the endoderm. The blastoderms are mounted in a hanging drop preparation, in Locke-Lewis solution, with the endoderm against the coverslip. The area pellucida is so thin that the endoderm, the vascular zone between the endoderm and mesoderm, and the mesoderm can all be analyzed with an oil immersion lens. The technique was described in 1920.

Blood-vessels begin by the differentiation of a new type of cell from mesoderm. This cell moves out of the mesoderm, develops a dense, basophilic, azurophilic cytoplasm and becomes physically more refractive than mesoderm. As soon as this cell divides, it shows one of its essential characteristics, namely, the tendency of the cells to stay together to form syncitial masses. These masses of cells put out sprouts by which they join similar masses to make a plexus. Both, while such solid clumps are still isolated, and after the plexus is formed, the cells become transformed into vessels by a liquefaction of the central part of the mass to make blood-plasma, while the periphery differentiates into endothelium.

There is a progressive differentiation of angioblasts, beginning in the periphery of the area vasculosa at the stage of two somites; the cells gradually appearing nearer and nearer the embryo, until, at the stage of five or six somites, angioblasts differentiate in the axial line of the embryo as forerunners of the endothelium of the heart and aorta. The heart, aorta and main vessels of the embryo differentiate *in situ* from angioblasts and increase by the addition of newly differentiated cells as well as by the cell-division and the sprouting of their endothelial walls. The amount of the differentiation of new cells grows less but at what stage it ceases is not known. Thus there is established the fundamental morphology of the vascular system.

Blood-vessels arise by the development of a new type of cell, the vasoformative cell of Ranvier, or the angioblast of His. This cell produces the first fluid of the blood; thus, endothelium is primary and blood-plasma secondary. Since there is a tissue fluid before angioblasts arise, endothelium is from the start a membrane between two different fluids, tissue-fluid and plasma. Moreover, the process of liquefaction is intra-cellular, that is, it can be seen in chains of single angioblasts which become vessels and it can also be seen to take place in the sprouts which are processes of cells, hence the lumen of vessels is embryologically intracellular and thus not a tissue-space.

In the living chick of the second day, it can be seen that both angioblasts and the endothelial cells give rise to red blood-cells. Erythroblasts begin in the chick in the vessels of the outer margin of the area opaqua in the stages of from 7 to 11 somites. In the area pellucida, where they can be seen in the living specimen, angioblasts differentiate during the stages of from 5 to 11 somites, while the vessels form and erythroblasts begin during the stage of from 11 to 14 somites. The heart begins to beat at the stage of 10 somites and the circulation starts when the chick has 16 to 17 somites.

During the past year, I have been continuing these studies on the living chick, and have found that it is possible to mount the entire blastoderm on a large cover-slip, one measuring 42 by 50 mm., throughout the third and fourth days of incubation. The cover-slips must be entirely free from grease or the membrane will not flatten out on the glass. From the fifth day on, the chick is too heavy to mount in the hanging-drop form, but if the specimen be transferred to a dish of Locke-Lewis solution, the amnion can be opened, the allantois pushed aside and the yolk-sac cut off close to the embryo and then spread out and mounted. The circulation, of course, stops but the membrane can be mounted and kept alive certainly for from three to five hours. So far, I have studied these living membranes only through the first seven days of incubation. It is an advantage to mount the chick with the membranes because the preparations are all fixed after the vital studies have been made and many of them are stained and mounted *in toto*. If the embryo has been left attached, it can be cut off in the alcohol and then the entire ectoderm can be dissected off from the area vasculosa. The specimen is thus made thinner and much easier to analyze. When the embryo has been cut away at the start, the ectoderm clings too closely to the specimen to be taken off and makes one more layer of stained cells in the final specimen.

I have found it a great advantage to study the embryos with a vital dye and add 1 to 3 drops of a 1 per cent aqueous solution of neutral red to 10 c. c. of the Locke-Lewis solution. making a dilution of possibly 1 to 10,000 of the dye, or less. This may be termed the physiological dilution of the dye.

After the specimens have been studied, they are fixed by floating the cover-slip, embryo down, on Bouin's picro-formol and then keeping them in 70 per cent alcohol until all the picric acid is removed. They are then stained in hæmatoxylin and counterstained in eosin with a little orange G. This fixation is excellent for the granulocytes, but quite worthless for the erythrocytes after the primitive stage. After fixation in Bouin's solution the young erythrocytes show only a wide-meshed reticulation having no relation whatever to any of the substances that can be made out in the living cell. No fixation of the blastoderms is adequate to follow the changes in the red cells, but they can be identified best after fixation in the vapor of formalin if not applied too long. I use it for from ten minutes to half an hour. In this the hemoglobin is well preserved but not the basophilic cytoplasm and indeed the earliest traces of hemoglobin which can be seen in the living cell by a distinct yellow color cannot be detected in the fixed specimens. Helly's fluid, which preserves the basophilic substance better, cannot be used, because the blastoderms float off from the cover-slip almost immediately and wrinkle so that they can never be studied as a section with an oil immersion lens.

In connection with the study of these blastoderms, a drop of blood is drawn from the vessels when the egg is first opened and used for a film. These films of blood are studied vitally by Pappenheim's method. They are made as follows: A clean glass rod is dipped into a dye and drawn across a perfectly clean glass slide. The two dyes which I have used are neutral red and brilliant cresyl blue, made up either in a 1 per cent aqueous solution or in a saturated alcoholic solution. The even film of the stain dries quickly and its strength is estimated by the color. The film must not be dense enough to stain any nuclei. A drop of blood is then drawn out with a fine glass cannula, placed on a cover-slip which is then inverted on the film of stain, ringed at once with salvoline and placed in a warm box. The vital stains develop slowly, on an average, in ten minutes, and if permanent preparations are wanted, the specimen is watched under an oil immersion lens until the staining is best, then the cover is drawn off from the slide and the film of blood counterstained with one of the blood stains. I have used Wright's eosin-methylene blue. In blood from chicks on the second and third days of incubation the amount of the specific substance of the red cells, that is, the megaloblasts, stainable in the vital dye is so massive, that it is necessary to differentiate the specimens after staining by Wright's method. This can be done in absolute alcohol and the decolorizing stopped with xylol. The white cells are so very unevenly distributed in the vessels of the early blastoderms that no film of blood represents adequately the amount of differentiation for the stage. Hence, each stage must be studied by both methods, by a survey of the total area pellucida and by drops of blood with specific stains.

In these studies, it can be seen that there are three different strains of blood-cells, first, those that arise from endothelium, which include both the red cells and the monocyte strain of the white cells; secondly, the granulocytes, and thirdly, the lymphocytes. The red cells begin to differentiate on the second day of incubation. On the third day the endothelium gives rise to the monocytes, that is, to the large mononuclears and the transitional forms of Ehrlich. In two different specimens I have seen an occasional monocyte on the second day, but the process becomes active only on the third day. The group of the monocytes of the blood is especially well illustrated by Pappenheim on his Plate 1,[22] as the third row of cells, and as the fourth and fifth rows on Ferrata's Plate 12.[23]

At the same time that the endothelium gives rise to the monocytes, namely, beginning on the third day, it gives rise to a much more numerous extravascular group of cells, identical with the monocytes, which are the clasmatocytes of the connective tissues. On the third day also the granulocytes begin to differentiate as a new type of cell from the mesoderm. These cells develop a specific type of granulation and wander into the blood-vessels. The third strain is the lymphocytes. These I have never seen differentiating in the wall of the yolk-sac; they begin to appear in the blood stream on the fourth day but do not become marked until the fifth and sixth days. It may be that they arise only within the embryo itself.

ERYTHROBLASTS

In these studies it has been possible to establish a criterion for a primitive red cell. As was discovered by Pappenheim,[24] the primitive red cell has a basophilic, azurophilic cytoplasm so finely granular as to appear like ground glass. Fixed to show this basophilic cytoplasm, the cell looks like a single angioblast. In the living cell a droplet of yolk is occasionally to be seen. If, however, one stains the cell supravitally with either neutral red or with brilliant cresyl blue, there appears a very massive granulation which at first completely fills the cell. This granulation is completely soluble in alcohol and in all of the usual fixatives; it disappears also in the vapor of formalin. If, however, it be stained with neutral red or with brilliant cresyl blue, it becomes insoluble in methyl alcohol and hence can be seen in films stained with Wright's eosin-methylene blue. For these double stains brilliant cresyl blue is slightly better. This special granulation is then very easy to bring out in films of blood. It is not so easy to stain in the total blastoderm, because the dilution which stains the neutral red granules of endothelium, the yolk and all of the stainable substances of the clasmatocytes, that is, the dilution of 1 to 10,000, is too weak to stain the granulation of the reds, but it does, nevertheless, make it just visible. The primitive red cell in the living blastoderm can be stained, however, by injecting the dye directly into the blood-stream; or if a drop of 1 per cent solution of neutral red is put on the blastoderm for a few seconds and then washed off with clear Locke-Lewis solution, the red cells show the stain well.

The question must come up as to whether the stainable substance actually exists in the living cell in some state from which it is precipitated by the dye, just as Mott has shown that the Nissl substance, in its stainable form, develops only as the nerve cell dies. Indeed, when Israel and Pappenheim [17] first described the vital staining of substances in erythrocytes, they

saw that the staining commenced only as the cell began to die. This may be true. The criterion I have used for the actual death of the cell is whether the nucleus stains or not, and this specific substance does stain in dilutions which do not stain the chromatin of the nucleus at all. The exact dilution necessary to stain it must be worked out.

For the permanent films of blood on the second and third days, the granulation is so dense that, after counterstaining with Wright's stain, the cells must be differentiated in absolute alcohol, the decoloration being stopped with xylol.

On the second and third days, the red cells being megaloblasts, both the granulation and the basophilic cytoplasm completely fill the cell. On the fourth day a narrow rim of clear cytoplasm appears in many of the cells making erythroblasts, showing hemoglobin around the edge free from granules, while the granules make a very dense rosette or wreath around the nucleus. These rosettes are very characteristic and in stained preparations they greatly obscure the nucleus. By the fourth day the red cells have no longer the somewhat uniformly round shape of the earlier stage and there is no longer a comparative uniformity in size, but rather there are many much larger forms together with many small irregular or oval cells. This period of great variation in size is a stage of active division of the cells as well as of growth of individual cells. In the small oval cells, the rosettes are oval and in division the rosettes divide, so that each daughter nucleus is surrounded by a wreath of granules before the cells separate.

On the fifth day a few of the red cells begin to show a diminution of the granulation, some of the cells grow much larger and the granules and rods begin to spread out into the cytoplasm, the cells showing the polychromasia and the reticulation which is known to be characteristic of the so-called reticular forms occuring in anæmias in human blood. By the seventh day there are large numbers of the cells in the reticular stage, while there are still many of the primitive cells and the wreath forms. Gradually the amount of the basophilic cytoplasm and of the vitally stainable granulation decreases while the hemoglobin increases. Finally, the basophilic cytoplasm disappears, but a small amount of the specific granulation remains in each erythrocyte. At the time of hatching and for three days afterward, all of the red cells in the circulating blood show from one to eight or ten vitally stainable granules. I have not carried the studies farther nor studied the stages between the seventh day of incubation and the time of hatching. It is clear, however, that one can work out the types of cells that are characteristic for each stage of the developing chick. Of course, at any stage there are some cells characteristic of preceding stages. On the second day only the primitive stage, the megaloblast, is present, and then, with a given increment of time, the different stages in the development of this specific granulation are added. When such a study has been made for a mammalian form and especially for the human embryos, as is now feasible, we shall be in a position to estimate just how primitive are the cells that appear in the circulation in anæmias.

The first account of the staining of the specific granulation of the red cells, which I have been able to find is in a paper by Israel and Pappenheim[17] in 1896, in which they say that if a few dry grains of neutral red are placed on a slide and used for a film of fresh blood, there will appear a granulation in some of the red cells just as the cell begins to die. In 1901, Bettmann[2] described the use of vital neutral red in the staining of red cells in pathological conditions, but did not discriminate between a staining of the nucleus and the specific granulation around it. Three years later, Rosin and Bigergeil[25-26] described the methods of vital staining and the dyes to be used, but it was not until 1907 that we have a clear account of the vitally stainable granulation of the red cells. In 1907 there appeared three papers in the Folia Hæmatologica, by Caesaris-Demel,[6] by Pappenheim[24] and by Ferrata,[12] in which the vitally stained granules are described and illustrated. Caesaris-Demel shows the stage of the wreath around the nucleus, not, however, in as primitive a stage as on the fourth day of incubation in the chick. He shows also the reticular stages and the final stage of a few granules. He distinguished between the deeply staining granules and the more faintly staining filaments.

The primitive basophilic cell, which is the first red blood-cell, was first differentiated by Pappenheim and called the megaloblast. It has a basophilic cytoplasm and a large nucleus, poor in chromatin, and a conspicuous nucleolus. It becomes an erythroblast and all the stages of the development of this cell, as far as concerns the decreasing of its basophilic cytoplasm, the increasing of its content of hemoglobin and the changes of its nucleus, have been worked out with the eosin-azur technique in final perfection by Pappenheim,[21-24] Ferrata,[12] Danchakoff,[5,6] Maximow[22-24] and Weidenreich.[39-41] The development of this cell with reference to its specific granulation is now necessary to complete its life history.

In the chick, it has been shown that all of the primitive blood-cells on the second day of incubation are megaloblasts which become erythroblasts as soon as a trace of hemoglobin can be made out. These cells are derived from angioblasts and from endothelium. As far as the specific granulation is concerned, the first stage, on the second and third days of incubation, has the granulation throughout the cell. From the fourth to the sixth day, there are the rosette or wreath forms in which the granulation is around the nucleus. On the seventh day most of the cells show the reticular form of the granulation. All of these stages show diffuse basophilia. With further studies it will be possible to tell just when basophilia and the reticular forms disappear in the majority of the red cells. At the time of hatching all of the cells in the circulation have acidophilic, hemoglobin-bearing cytoplasm with a few vitally stainable granules. It is, of course, clear that at any stage in development, while the majority of the cells are in a specific phase, a few of the earlier types may be found. In normal human blood about one per cent or less of the erythrocytes show a few vitally stainable granules.

The question which must come up first in connection with this granulation is its relation to the so-called basophilic punctation, and both Pappenheim and Ferrata agree that these two substances are entirely different. The basophilic punctation stains in azur after fixation and Ferrata [23] thinks that it is an abnormal clumping "conglobation" of the azurophilic cytoplasm, and he shows the gradual production of the punctate forms in red cells after experimental lead poisoning on his Plate VIII. It is thus easy to see why basophilic punctation does not occur in embryonic blood, whereas the vitally stainable granulation is specifically characteristic of development.

Another point in which this specific granulation may prove of interest is that it offers a chance to study the development of hemoglobin in the cell by testing the granulation for the presence or the absence of iron. Both the azurophilic cytoplasm and the granulation disappear as hemoglobin develops, but the granulation alone is characteristic of the red cell as distinct from all other blood cells.

In the developing blood there are always a few cells containing the Howell-Jolly bodies. These are fragments of nuclei staining just like chromatin, which were discovered by Howell,[16] in 1890, in a study of the blood of the cat after hemorrhage. Of course the corpuscles of the chick are all nucleated, so that the question of the extrusion of nuclei does not come up, but an occasional cell, from the very beginning of the formation of blood on the second day, shows a fragmented nucleus. I interpret such cells as dead. They are to be found in the early blood islands before the cells become free and are very interesting as showing that cell death occurs in the early stages of marked cell-division and growth.

ORIGIN OF MONOCYTES AND CLASMATOCYTES FROM ENDOTHELIUM

The separation of the clasmatocyte as a distinct type of cell of the connective tissues is due to Maximow.[20] He showed that by introducing two sterile cover-slips under the skin in rabbits, one could separate three types of cells by the speed with which they passed between the covers, leucocytes appearing first; a special cell, the clasmatocyte, wandered in during the first nineteen hours and the fibroblast in from two to four days. Then he showed that the clasmatocyte was specifically sensitive to neutral red,[21] while Bouffard,[3] Goldman [14,15] Evans,[7-11] Schulemann [7-9] and a large group of workers have demonstrated that it is the cell of the connective tissues most specifically differentiated to phagocytize and store particulate matter. The specific reaction of this cell to vital, neutral red is that the dye stains certain granules of the cell and certain large fluid spheres which are called vacuoles, the so-called "neutral red granules and vacuoles" of Lewis and Lewis.[13] These vacuoles are organs into which the cell passes phagocytized particulate matter. In the vacuoles the fine particles which the cell has taken up become clumped and, as Evans and Scott [11] have shown, may even be re-crystallized.

Aschoff and Kiyono [1] then showed that an identical reaction to a vital dye could be obtained by certain cells of the blood, namely the group Naegeli [20] has called the monocytes, which are the large mononuclear and transitional forms of the Ehrlich school. Thus they distinguished and related histiocytes of the blood and histiocytes of the connective tissues. Moreover, they regarded the histiocytes of the blood as of endothelial origin.

Pappenheim and Ferrata have illustrated the separation of the monocytes, the leucocytes, and the lymphocytes on purely morphological grounds, believing that there is a common stem-cell, a hypothetical hæmatoblast for them all. Aschoff and Kiyono separate the monocytes, calling them histiocytes of the blood, on a physiological basis, and I think that I can demonstrate on a fundamental embryological basis that the monocyte and the clasmatocyte are identical cells, derived from endothelium, thereby making one of the three great groups of connective-tissue cells that contribute to the blood.

If a blastoderm of the third day of incubation be stained in vital neutral red, the endothelium stands out with numerous granules staining in the dye which are both around the nuclei and scattered in the thin periphery of the cytoplasm. The endothelium of the capillaries and the veins often becomes reduplicated. Endothelium is more refractive than mesoderm, and this property, as well as the granules stainable with neutral red, characterizes both of these layers of endothelium. One of the cells of the inner row can then be seen to enlarge, protrude into the lumen and develop the vacuoles which are characteristic of clasmatocytes. The periphery of the cell then puts out a film of cytoplasm in which there is a central process more refractive than the rest, simulating a flagellum, and these films are in constant motion. In fact the eye is attracted to these cells both by the stained vacuoles and by the motion of the peripheral films of cytoplasm. Such a cell then gradually becomes free. The characteristic motion of the peripheral films continues, keeping the surrounding fluid moving, though the cell itself shows very little locomotion. In the study of the origin of the red blood-cells on the second day of incubation (Sabin)[17] it was noted that the erythroblasts formed great clumps of cells attached to the inner surface of a complete endothelium. The monocytes, on the other hand, differentiate and drop off as single cells, leaving the original endothelial cell from which they came as the wall of the vessel.

An endothelial cell may become phagocytic while it is yet in place, for I have seen them with red cells engulfed just as Maximow [20] shows for a mammal in his Fig. 4 on Plate XVIII. This means that an endothelial cell which is actually a part of the wall of a vessel, not one of the reduplicated forms already on the road toward becoming free, may be phagocytic. That is to say, endothelium is itself phagocytic as well as having the power to give off free cells which are phagocytic. In the same figure quoted above, Maximow shows three free monocytes, very characteristic, labeled Edph. He recognized them as desquamated endothelium but did not identify them as monocytes. In fact all of the early stages of the develop-

ment of blood are beautifully illustrated by the two plates of Maximow in this same article.

While these few cells are getting free in the lumen of the vessel to make the monocytes of the blood, the outer row of the reduplicated endothelium divides rapidly in irregular patches, making the outlines of the vessels, both capillaries and veins, have an exceedingly irregular contour, very different from the smooth contour of the earlier capillaries and from the wall of the omphalomesenteric arteries which now have a single layer of smooth muscle. The clumps of cells along the outer wall of the vessels develop the vacuoles characteristic of clasmatocytes and become free as clasmatocytes. Many hundreds of the extra-vascular cells are formed from the endothelium to one intra-vascular. The extra-vascular forms tend to be larger and have larger vacuoles, but I have seen one of the large cells wander into a vessel. The original endothelium has granules that stain in neutral red; it may also have vacuoles. The free cells all have both vacuoles and granules and a differentiation of the periphery of the cell into motile films. Studied with vital neutral red, the monocytes and the clasmatocytes are conspicuous because they are stained.

Thus, in the early chick, endothelium gives rise to two groups of cells, the megaloblasts which develop hemoglobin and become erythroblasts and a strain of cells termed histiocytes by Aschoff. The extra-vascular histiocytes have been termed clasmatocytes, the intra-vascular ones monocytes. They are identical and are specifically differentiated along the line of phagocytosis. They take up particulate matter and debris in solid form which they segregate and store in certain preformed vacuoles filled with fluid. They do not store this insoluble material permanently because it is gradually returned to the circulation and excreted by the kidney. So they represent a mechanism for taking care of foreign matter in excess of the amount that the body can excrete at the time. In the blastoderms from the third to the seventh day there is comparatively little differentiation of new angioblasts in the area pellucida. In fact, in about fifty specimens, I have found only three masses of solid angioblasts. The hollow isolated vesicles made from these solid masses are, however, more numerous, indicating that this stage lasts longer than the solid stage. In one specimen of the third day of incubation there was a long mass of solid angioblasts which started to liquefy to form a vessel, and while the center of the mass was liquefying to form a vessel, two cells wandered off from the periphery as clasmatocytes. Thus angioblasts can also give rise to clasmatocytes.

If one takes the group of monocytes as they are shown in the third row of Pappenheim's Plate I,[10] or the fourth and fifth rows of Ferrata's Plate XII,[12] it will be seen that they include all of the large mononuclear forms and the transitionals of the circulating blood. Both of these types can be seen early in the chick coming from endothelium; an endothelial derivative which is larger and less vacuolated is the mononuclear cell, a smaller and more vacuolated type, the transitional. The transitional is thus shown to be a finished type of cell like the cell of the adult form, for which the term transitional is therefore a misnomer. The large mononuclear type always has an excentric nucleus; it is distinguished most easily in the films of blood, stained with brilliant cresyl blue and counterstained with eosin-azur. It lacks the specific granulation of the erythroblast and has a very clear distinctive blue cytoplasm in Wright's blood-stain. With the group of the clasmatocyte in the connective tissues, Maximow divided the cells into resting and active cells. With the group of endothelial derivatives in the blood, it is not wholly clear whether the larger or mononuclear forms are resting or are old forms. In the embryo, the large mononuclear forms appear less specifically differentiated. Both forms can be seen in the living chick to come from endothelium, the transitional cell being developed specifically along the line of phagocytosis and storing of particulate, solid matter and possessing a certain type of motion of the cell *in situ* and very slow locomotion like the clasmatocyte of the connective tissue.

The Granulocytes or Leucocytes

On the third day, also, granulocytes begin, represented by the cells which are analogous to the neutrophilic myelocyte of mammals. In the chick the granulocyte with fine granules is pseudo-eosiniphilic. The first sign of the beginning of the granulocytes is that a cell appears close to a vessel which cannot be distinguished from a single angioblast. I have not found in these cells any substance stainable in neutral red except the specific granulation which stains paler than the granules of the endothelium, but am not yet entirely sure that this will be a sufficient distinguishing mark between this cell and a single angioblast. When, however, such a single cell divides, there is no longer any difficulty, because two angioblasts stay together while two granuloblasts separate. This criterion is not adequate when one has only sections, but in watching the living membranes or in studying them after fixation, where every cell of an entire area can be seen in its relations to other cells, it is sufficient. Such material has obvious advantages over sections. Thus, from one cell comes a clump of four or more cells with a dense azurophilic cytoplasm, the stem cell of the monophyletic school, lying near a vessel. These cells then show the following changes. The nuclei become excentric, while the center of the cell is occupied by the centrosphere made very obvious by the development of fine granules, staining pink in neutral red, always arranged in a crescent around the centrosome. Thus, there is a nucleus on one side, a clear spot in the center of the cell and on the other side this crescent of fine granules. The granules are entirely motionless at the start, there is none of the active streaming of the granules which is always associated with amœboid movement and which must be associated with a fluid state of the cytoplasm. The cell itself, however, does move, but very slowly, directly toward the vessel. One of the cells reaches the wall, half-way between two endothelial nuclei, and then one can see the wall bend inward, until finally the cell enters the lumen. The rest of the clump line up behind the first and also pass in. Thus, these granulocytes show a specific chemotactic reaction at once. Throughout these early

stages the granules are arranged characteristically around the centrosome. Thus, the specific granulation of the red cell is arranged around the nucleus, of the granulocyte around the centrosome, while the granules of the endothelial cell are scattered throughout the cell. Even in these early stages the nuclei of many of these cells become indented, the concave side always being toward the centrosome; thus, the primitive cell may soon be regarded as a leucocyte.

In the case of the monocytes and the clasmatocytes, both of these cells can be readily found differentiating and dropping off from the endothelium, but no relation to endothelium can be made out in the case of the granulocytes. They are near vessels but never form a part of their wall. It was shown by Danchakoff [8] in 1908 that the granulocytes are extravascular in origin.

There are no eosinophilic cells on the third day. The eosinophilic granule of the chick's blood is in long rods. During the first seven days I have seen only a few in the circulation and have not found them differentiating in the area pellucida. Probably further study will bring them out, since they are known through the work of Danchakoff [8] to develop in the area opaqua of the yolk-sac. The mast cells I have not seen at all in the first seven days, and Maximow [24] found that they develop late in mammals.

From these observations one may offer the theory that the two stem cells, the angioblast with its power to give rise both to red cells and to histiocytes in the larger sense, and the granulocyte are cells whose common ancestor is a mesenchyme cell instead of a differentiated stem cell or "hæmatoblast." In other words, the cells of the blood are not so sharply marked off from the cells of the connective tissues as to have a specific, common stem cell. At least one would have to prove that the differentiated cells which normally made the syncitial masses of angioblasts could be made to develop granulocytes. The argument for the mesenchyme cell as the stem cell, for the three distinct strains of cells which contribute to the blood, is that three such groups can be isolated embryologically and that they correspond with a functional classification. At least one may say that no common differentiated stem cell has been adequately demonstrated.

LYMPHOCYTES

In these studies of the development of blood in a living form the account of the origin of the lymphocytes is very incomplete. The lymphocytes make a group of cells ranging in the mammal from the size of a red cell up to cells twice the size. Likewise in the chick the lymphocytes are the smallest cell. When the cell first appears, all of them are of the small size. The cell has a characteristic nucleus and its cytoplasm contains a few azurophilic granules, discovered by Michaelis and Wolff.[25] The living cell has a nuclear membrane which is more distinct than in any other cell, but that this criterion is a difficult one to go by can be realized readily in connection with the fact that all nuclei become distinct as a cell dies. The cytoplasm of the lymphocytes contains but few granules and they do not stain readily in neutral red, but can be made to by increasing the amount of the dye or the time of staining. From these facts it is less readily discriminated than the other types. The reactions of lymphocytes in tissue cultures have been described by Lewis and Webster.[19] In Wright's stain, the early lymphocytes are exactly as distinctive as in adult blood. The first forms are of the small variety. I have seen a few on the fourth day, more on the fifth and sixth. In the blood smears, they occur in small clumps. The chromatin of the nuclei is very dense and has a peculiar violet reaction with azur-eosin. I have never found any indication of their differentiation in the area pellucida, thus it may be that they form only within the embryo itself rather than in the yolk-sac. However, a more extensive study of the yolk-sac may bring them out. All of the evidence from the study of this cell in the adult is that it differentiates extra-vascularly from reticulum. The only evidence, then, of significance in these studies in regard to this cell is that it occurs later than the other two groups and hence should not be regarded as a stem cell. Thus, from these studies, I would stress the use of the three names of white cells as specific for the three distinct groups, the leucocytes, the monocytes and the lymphocytes.

In these studies it is very plain that each white blood-cell, as it first appears, is differentiated. The red cells pass through a long stage of maturation. The first megaloblasts can be told as early stages of the red cells by a specific granulation, but the cell itself passes through a long series of stages, the erythroblasts, before it is the erythrocyte of the adult blood. The monocytes are a phagocytic type, like the cells of the adult, before they leave the wall of the vessel, the endothelium itself being phagocytic; the granulocytes develop their specific type of granulation early and soon begin to be leucocytes, and thirdly, the first lymphocytes are distinctive. When, however, all of these cells begin to divide, discrimination between all of the young cells is by no means easy, as the entire history of hematology attests. From this it can be seen readily that one must continue these studies of the development of blood in these living forms, watching especially the young cells just after division in all the stages of incubation, before one can adequately master all of the types of cells that are to be seen in bone marrow. In a drop of blood taken on the third day of incubation it is possible to tell all the cells apart, later it becomes most difficult. It is the study of the maturation stages of each group of cells by means of the eosin-azur technique that has been the great contribution of the monophyletic school. To this study must now be added certain specific criteria that come out through the method of applying dyes to living cells and we must now follow the stages of the cells with these vital dyes through the different embryonic periods.

The postulation of three strains of blood-cells on the basis of embryology fits in with the functional groups as we now know them. The endothelial or angioblastic group represents first the hemoglobin-bearing cells, and secondly, that group of the blood-cells which exhibit a special property of endothelium, namely, phagocytosis. The monocytes have this power of phagocytosis, they possess a peculiar type of motion *in situ* with very slow locomotion. The granulocytes possess a high

degree of amœboid motion, with speed and a flowing of the granules. They respond to chemotactic influences, are also phagocytic and have functions probably related to their specific granulations. The lymphocyte strain, as Murphy[26-29] has shown, are separated off physiologically by their being more sensitive to X-rays and to the emanation of radium than other normal cells. Moreover, he has shown that they are related to immunity toward certain forms of tumors as well as to certain types of infection.

The study of the blood-cells is a part of the study of the cells of the connective tissues. The erythrocytes are the only type that function only within the vessels. Of the other group from endothelium, the histiocyte in the larger sense, the vast majority make the clasmatocytes of the connective tissues, which are the mononuclear forms and the actively phagocytic forms of sub-acute infection, the resting and active wandering cells of Maximow. A few of this group make the monocytes, that is, the large mononuclear and transitional forms of the blood.

Of the granulocytes, which all differentiate extra-vascularly, the neutrophilic leucocytes pass into the vessels in largest numbers. Of the eosinophiles very many remain in the tissues while the mast cells never enter the vessels in most animal forms. By mast cell is meant a cell of the connective tissues occurring along vessels, along nerves and between muscle fibers, having a special metachromatic, basophilic granule. The so-called mast cell of human blood has been shown by Weidenreich,[30-31] to be a degenerating cell without any centrosome. The lymphocytes are for the most part extra-vascular, arising in the lymph glands and in the follicles of the spleen and in very numerous follicles in the various organs either associated with lymphatic capillaries or not. Thus, the differentiation of three strains of blood-cells, the endothelial strain, the granulocyte strain and the lymphocyte strain, that can be made out in the early stages of the chick embryo, can be shown to correspond with a functional grouping as far as we yet know the functions of the types of blood-cells. Moreover, the origin of the cells of the blood can be shown to be but a part of the study of the great groups of wandering cells of the connective tissues, the one type which functions only intra-vascularly being the erythrocyte.

The method of studying blood with vital dyes, beginning with the stages of the embryo when the cells first appear gives a very great advantage in following the maturation of specific cells and gives a chance of analyzing the complicated young forms which it is necessary to recognize in order to understand bone marrow.

The group of the red cells is characterized by a specific granulation stainable in certain vital dyes, possibly one should say precipitated by these dyes. This substance is at first throughout the cytoplasm, then in a wreath around the nucleus, then a reticular form and finally in scattered granules or droplets. The arrangement of this granulation around the nucleus should be stressed, although the substance is of cytoplasmic not of nuclear origin. Red cells with nuclear fragments, Howell-Jolly bodies, can be shown to be dying cells.

The strains of white cells, clasmatocytes and monocytes, that come from endothelium, are characterized by certain granules and vacuoles stainable in very dilute neutral red. They are scattered diffusely throughout the cells. The granulocytes are characterized by the arrangement of their specific granulation with reference to the centrosome. The lymphocytes are less sharply characterized morphologically, but have somewhat distinctive nuclei and granules stainable in azur.

This work is a part of the new subject of experimental cytology which seeks to analyze cells by means of specific criteria and to use these criteria to study the reactions of cells to normal and abnormal conditions.

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SOME OXIDATION MECHANISMS OF THE CELL[1]

By F. GOWLAND HOPKINS

Oxidation in the living tissues offers to biochemistry what is perhaps its chief problem. The living cell receives a supply of molecular oxygen; it also receives materials which it ultimately oxidises. Yet towards these materials molecular oxygen is indifferent. These facts define the problem. The biochemist cannot avoid the task of trying to solve it.

I propose to devote two lectures to a consideration of certain limited aspects of the subject, feeling sure that you will understand the necessity for limitation. On the present occasion I shall deal with a special type of chemical mechanism, concerned directly or indirectly with oxidations in the cell, which has been the subject of recent investigations. The mechanism in question is found in nearly all animal tissues, and is active in certain tissues of the plant.

The second lecture will deal with a highly specialised tissue—striated muscle. It will be devoted to a discussion of the incidence of oxidations; to their position, that is, in a chemical sequence. Incidentally it will deal with the influence of oxygen upon equilibrium in the tissue. A prominent point in all that I shall have to say is the importance of the events which precede actual oxidations.

It is always desirable, before dealing from any standpoint with the functions of oxygen in living processes, to have in mind the fact that life in certain associations can dispense with it altogether. We know that certain living organisms, usually of a kind adjusted to the conditions of parasitism, are maintained without oxygen, and among them are not micro-organisms alone but highly differentiated animals, such as intestinal worms. The energy requirements of such animals are met by the occurrence of exothermic chemical processes which involve the employment of free molecular oxygen. Though it is quite possible that life began in the sea at a time when the available oxygen in the atmosphere was much less than at present, we are perhaps not justified in assuming that these living processes without oxygen are more primitive than those in which it is employed. Nevertheless, the functions of oxygen in life do represent something superadded to such phenomena as are entirely essential. It is important to the line of thought which you will be asked to follow that two aspects of this relation should be emphasized. The first is that living tissues, even those of the higher animals which are fully adjusted to employ oxidations to advantage, do not necessarily lack the power of extracting energy from materials in the absence of oxygen. Such methods of obtaining energy—this being the second point for emphasis—are, however, relatively, extravagant. Perhaps the most important function of oxygen in living processes is to establish economy of material.

[1] Lecture I of the Herter Series delivered before The Johns Hopkins University, April 12, 1921.

Much intellectual ingenuity has been expended in the endeavor to throw light on the oxidative mechanisms of the cell and not a few theories have been propounded. Those which, from one standpoint or another, have visualised the activation of molecular oxygen or the influence of special catalysts within the cell have stimulated experiment and assisted progress, even when they have, as theories, failed to survive.

Certain views, however, which were developed during the last third of the nineteenth century have, in my opinion, inhibited productive thought and discouraged experiment. I allude to those views which picture the dynamic events associated with life, including of course the essential oxidation, as occurring in a particular kind of large chemical molecule within the cell. During phases of construction or reconstruction, both the oxygen and the materials to be oxidised are supposed to be built up into the structure of this living molecule. Alternating with such phases, and particularly encouraged by stimulation, are phases of breakdown during which the products of oxidation emerge from the molecule, accompanied by liberated energy. Such views were expressed vaguely in the *inogen hypothesis* of Hermann and more objectively in Ehrlich's *side chain theory* and Verworn's *biogen hypothesis*. All these imply that those parts of a living cell which manifest the essential attributes of life are made up of a number of like molecules, each of which is in some sort a unit of living matter. When they remain quite vague in detail, such conceptions offer no aid to thought; when details are supplied, they attribute qualities to the living unit which can hardly belong to a single molecule, if we use the word in any ordinary sense as denoting a system in which atoms or atomic groups are associated by valency.

The alternative view is that the living cell (or an equivalent structure) is itself the unit. The manifestations of life viewed from this standpoint depend upon changes undergone by diverse molecules of a kind which need not elude ordinary chemical studies. Such molecules, of an order common to other non-biological chemical events, take part in diverse chemical reactions which, though they occur in a colloidal milieu, are themselves ordinary in the sense that they can—individually at any rate—be studied by the recognized methods of chemistry. On this view, the essence of what is peculiar to the cell as a chemical system lies not in the nature but in the organization of its processes.

To explain the remarkable organization called for, is, and doubtless will long remain, a very hard task. Nevertheless, the view just mentioned is the one which is supported by all the experimental facts that we possess, and is one which will most surely stimulate further experimentation. It is to be observed of all theories based upon the conception of a living molecule or biogen that they make no real attempt to explain the unknown in terms of the known. They escape from the hard task of analysis by falling back upon the properties of an entity to which, since it is wholly imagined, any attributes may be ascribed. They form, I think, little more than comfortable couches whereon the mind may rest and forget the task which after all remains ahead.

It seems to me that the two opposed views that are before us, the one conceiving that all the chemical events in the cell occur within the structure of a particular and highly exceptional kind of molecule, the other looking to the progress of ordinary chemical reactions, with associated changes in the physical equilibrium of colloid systems, as being the essential basis of events, may be tested by the application of one particular criterion. The former view, if it is to help thought at all, involves inevitably a belief in intramolecular oxygen; in the building up of oxygen into the living complex upon which it confers instability. If oxygen be not "assimilated" before it functions; if, that is to say, the oxidations in living tissues proceed under the influence of an immediate and contemporary oxygen supply, then it is sure the biogen hypothesis loses almost the whole of any value it might otherwise possess. I shall deal later—more particularly in my second lecture—with evidence which provides this criterion. It leaves no doubt in my opinion that the conception of intramolecular, assimilated, oxygen is without basis in biology.

I will ask you to believe that this preliminary reference to considerations which are vague is not without point. Had we to admit that conceptions such as that of the living biogen are in any sense necessary to biological thought, I personally feel that the experimental findings which are to form the main theme of this lecture, and all others of a similar order, would lose most of their significance. They would refer only to events in the cell which are purely superficial. The really significant events occurring in an unapproachable molecule would evade such experimental attacks altogether. I am to deal with certain special facts alone, but in order that these may fit into their proper place it is necessary for me first to go over certain more familiar ground.

It is right to speak of Schönbein as the real pioneer in experimental attacks upon our problem. This distinguished observer (I am quoting the late J. H. Kastle) seems to have been the first to recognize clearly that in the final products of chemical combination, particularly those in which oxygen is concerned, we see, as he puts it, only the closing scene of a chemical drama which is in reality composed of several intermediate acts, and that for a correct understanding of such processes it is quite as essential to know these intermediate acts as it is to know the beginning and the end. All illuminating studies of metabolism have been inspired by this belief.

The study of tissue oxidations thus received its impetus under the influence of Schönbein in the middle of the last century and, since he wrote, it has on one line or another continued. But really significant data leading to modern views may be said to have first arrived with studies of autoxidation as made by Engler and Bach and extended into biological problems by Bach and Chodat. The accepted views based upon the work of these authors were foreshadowed by Traube, though upon small experimental basis.

Certain substances—though neither the foodstuffs nor their immediate derivatives—are capable of spontaneous oxidation when in contact with molecular oxygen, and this at ordinary temperatures. When such autoxidation occurs it can be

usually shown that the oxygen is taken up, not atom by atom, but as a molecule still partly intact, only one of the two bonds of the original molecule being opened up. Thus if R represent the autoxidisable substance there is first formed an association of the peroxide type, $R\begin{smallmatrix}O\\|\\O\end{smallmatrix}$. Ostwald and Bach have pointed out that, as a matter of fact, such a compound must, under the circumstances of autoxidation, be in all cases first formed, even if it have but an evanescent existence. By Ostwald's Law of Successive Reactions the less stable state represented by RO_2+R must precede the more stable state $RO+RO$.

If autoxidation is thus always associated with peroxide formation, its occurrence in the cell even on a very small scale must be a significant thing, because once peroxide oxygen has appeared it may be transferred to substances not themselves autoxidisable. Autoxidisable substances can act catalytically or as parts of a catalytic system. If such are present in the cell, however low their concentration, we have at least the beginnings of a mechanism for tissue oxidations. I need hardly remind you that an amount, probably minute, of material capable of autoxidation is known to exist in living tissues, or at least in some of them. Endowed with the power of combining with atmospheric oxygen to form peroxides, such substances constitute the so-called oxygenase factor in oxidase systems. A second factor in these systems—the peroxidase—in some way accelerates the transference of the peroxide oxygen to substances by themselves incapable of taking up the original atmospheric oxygen. The nature of peroxidases and how they act need not trouble us here. We may rest content with the close analogy between their influence on the activity of organic peroxides (oxygenases) and that of iron on oxidations by hydrogen peroxide.

These oxidase systems are doubtless familiar to you, and I need discuss their nature no further, though I would like to remind you of an interesting fact which we owe to my colleague at Cambridge, Mrs. Onslow[2] (formerly Miss Muriel Wheldale). She has shown that the autoxidisable substance which functions as the oxygenase in many plant tissues is catechol, or substances containing the catechol grouping; the ortho relation of two hydroxy-groups in these compounds makes them prone, apparently, to peroxide formation.

The oxidations which have been observed to occur under the influence of these oxidase systems are not such as involve deep-seated change or massive energy discharges in the cell. They have been thought of as being mainly protective, rather than metabolic, in function. It would be wrong, however, to forget that their activity as observed in the test-tube may be more limited in its effects than when it is displayed as part of an organised sequence of events within the cell. It is sure that cell oxidations involve the organization of various agencies. The recognition that one of these agencies secures the availability of peroxide oxygen undoubtedly constitutes a step towards the solution of the problem with which we are dealing.

But I am more concerned to remind you of other agencies. Oxidations may occur without the aid of atmospheric oxygen.

In a sense it is true to say that every oxidation is associated with reduction. Any agent which oxidises is itself reduced. Sometimes the reduction process seems to be the essence of the phenomenon, as when hydrogen is transferred from one substance to another; the latter being thus reduced. By long established custom, however, and with full justification we speak of the substance which loses hydrogen as being itself oxidised. I would not refer to so elementary a point were it not so important in all that follows.

The simultaneity of oxidation and reduction may present a prominent aspect of the phenomena when the molecule of water is concerned in chemical changes. It is of much biological importance to realize that oxidations and reductions in the living cell may follow upon a simultaneous transference of the ionic components of the water molecule. Water after all is by far the most abundant reagent present in the cell. In following up this aspect of events we shall be considering tissue oxidations which proceed, up to a certain point at least, without the intervention of atmospheric oxygen.

It will be convenient here to note a point in nomenclature. Engler gave the name of *oxygen acceptor* to any substance which may take up the oxygen of peroxides formed in autoxidation. When reduction and oxidation occur together, as in the case we are about to consider, we speak on the one hand of the *hydrogen acceptor* and on the other of the *oxygen acceptor*. The terms are convenient.

Bach[3] has very fully studied reactions which he calls *hydrolytic oxidation-reduction reactions*. In these under the influence of such catalysts as metals of the platinum group the reduction of one substance by the hydrogen ions of water is associated with the simultaneous oxidation of another substance by the hydroxyl ion. Such a case, with an organic catalyst taking the place of the metal, is the well-known Schardinger reaction induced by an enzyme present in fresh milk. Milk neither reduces methylene blue nor oxidises an aldehyde, but if both are present, the former substance acts as a hydrogen acceptor and the latter as an oxygen acceptor, and simultaneous reduction and oxidation occur. Bach has shown that in the tissues there is an enzyme identical with, or similar to, the Schardinger ferment of milk. It is important to remember that in reactions catalysed by enzymes of this class—at any rate if Bach's view concerning their action be correct—a hydrogen acceptor and an oxygen acceptor must both be present. It should be noted that Schardinger's ferment plus an oxygen acceptor would constitute a system with the properties of a *reductase* or *reducase*. On the other hand, combined with a hydrogen acceptor the ferment would show itself as a catalyser of anaërobic oxidations.

[2] Muriel Wheldale Onslow: Bioch. Jour., 1919, XIII, 1, *Ib.*, 1920, XIV, 535 and 541.

[3] Bach: Berichte, 1909, XLII, 4463; Oppenheimer's Handbuch, Ergänzungsband, 1913.

Having just now referred to the use of methylene blue as a hydrogen acceptor I should like to add just a word concerning it.

In classical experiments, Ehrlich, as you are well aware, injected into the living animal dyes of this class, which became colorless on reduction, to test the oxygen affinity of the tissues. The method as used by him has suffered much criticism of late, and the results it yields are doubtless open to misinterpretation. But, like other technical methods liable to error in uncontrolled experiments, it may yield valuable evidence when a proper control is provided. Such an informal use of methylene blue injections was made by the late Dr. Christian Herter[4] when he showed that depression in the temperature of the body diminishes the intensity of oxidations in the nerve cells of the brain, while fever increases it. It is a source of satisfaction to know that the facts I am to put before you are very closely related to matters which awakened interest in Dr. Herter's mind during the later years of his scientific life.

I shall have to refer a good deal to the use of methylene blue; but only when employed in the test-tube as an indicator of the fact that labile hydrogen is present in a particular chemical system. When the dye is decolorized we know that hydrogen has been transferred to its molecule. We assume, generally with full justification, as we register the progress of decolorization, that the same reduction will happen in the cell when a suitable physiological acceptor of hydrogen is present. The dye is merely a representative of such.

Returning to the tissues I must next point out that Wieland[5] claims for them the possession of a catalytic mechanism of which the influence may be general enough to include the supposed work of those already mentioned.

This author sees the essential cause of certain typical biological oxidations in the activation, not of oxygen, but of hydrogen. A proof that this activation occurs would render unnecessary, he thinks, any attempt to give a general application to the Engler-Bach peroxide theory, while it should modify somewhat the angle from which the hydrolytic oxidation-reduction processes are viewed.

Starting with the conception of a catalyst which acts as a "dehydrase" and confers lability on hydrogen atoms, the assumption that oxidases, reductases and "mutases" separately exist becomes from Wieland's standpoint superfluous.

His conceptions are especially supported by a careful quantitative study of the action of Schardinger's ferment upon salicylaldehyde.

The aldehyde is assumed to act in the form of its hydrate. The enzyme activates two of its hydrogen atoms which are then made available for an acceptor. This acceptor may be (1) molecular oxygen, (2) such reducible substances as are represented by methylene blue, or, in a particular case the acceptor may be (3) a molecule of the aldehyde itself in its unhydrated form. As an end result we have, in case (1), a frank oxidation of the aldehyde; in case (2), an observed reduction which, however, is associated with indirect oxidation of the aldehyde; in case (3), on the other hand, the Cannizzaro reaction—the so-called "mutase" effect.

The following scheme illustrates these relations:

$$R-CH\begin{cases}OH \xrightarrow{1} H_2 \quad O=H_2O \quad +\\ \xrightarrow{2} H_2 \quad A=AH_2 \quad +\\ OH \xrightarrow{3} H_2 \quad R.COH=RCH_2OH+\end{cases} R.COOH$$

Whether any of these end results is attained, or a mixture of all three, depends—as Wieland shows—upon the concentration, and speed of reaction of the various acceptors. The most important hydrogen acceptor for energy production in the cell is, clearly, oxygen; but the reduction of other acceptors may be equally important for other aspects of cell activity.

I do not myself think that the existence of a catalyst capable of securing the shifting of hydrogen on these lines should be thought to lessen the importance of those oxidase systems which we earlier considered. After all there is direct evidence for the formation of peroxides in the tissues, and in the organization of events it is likely that more than one mechanism is used, even to attain what may seem, superficially, to be similar results. Wieland, as we have seen, assumes in the particular case which serves as an examplar of a "dehydrase" reaction, that the aldehyde reacts as a hydrate. The necessity for this assumption shows after all that the special action of the enzyme as conceived need not differ in essentials from the hydrolytic oxidation-reduction of Bach. Wieland's experiments are very instructive, however, and I have found his views to be very helpful in dealing with facts which I shall later put before you.

Thunberg[6] has accepted Wieland's conceptions and in a paper recently published he has used the methylene blue technique in striking experiments which supply evidence—as he believes—bearing on the probable nature of intermediary metabolites. He suspended muscular tissue, deprived of its power of reducing methylene blue by thorough washing with water, but still containing the catalyst, in a weak solution of the dye. Small quantities of the substance under investigation were then added. The methylene blue acted, of course, once more as the hydrogen acceptor. If the second substance were capable under the influence of the enzyme of yielding hydrogen, acting, that is, in Thunberg's terminology as a hydrogen "donator," then, the system being completed, the dye was decolorized. Thunberg speaks of the ferment as a hydrogen "transportase." The substances which proved themselves to be donators were certain organic acids like succinic, malic, lactic, oxybutyric, and others; also amino acids like alanine and glutamic acids. They formed only a limited proportion of the large number of substances tested, and the fact that their structure evidently fitted into the system established by the tissue ferment and a hydrogen acceptor yields, in Thunberg's view, presumptive evidence that they represent actual tissue metabolites. The technique of these experiments was a little less simple than my description would imply. There was evidence of the existence of specific catalysts, each

[4] Herter: Amer. J. Physiol., XII, 128.

[5] Wieland: Berichte, 1914, XLVII, 2085.

[6] Thunberg: Skand. Arch. Physiol., 1920.

related to one substance rather than to others and, in a given case, the tissue might have to undergo preliminary treatment in order to isolate more or less the activity of the particular enzyme concerned.

I have dealt rather fully with this recent work which seems to establish the importance of the conception of acceptors, and especially of hydrogen acceptors when they co-exist with substances to be oxidized. I have done so because the facts, if not the views, involved prepare the way, I think, for the presentation of some experimental results obtained by myself which I wish to put before you. You must not judge of the significance of the doctrine of the activation of hydrogen without remembering that it supposes the ultimate fate of the hydrogen, labilized and transported, is to be itself oxidized. It ultimately, if not immediately, appears in such form or relations that it becomes amenable to oxidation by molecular oxygen. It is interesting to note that these recent views involve a certain divagation in the paths, and a difference in the mechanisms whereby carbon and hydrogen are respectively brought to oxidation. If, to take a single merely illustrative instance, lactic acid be supposed to act first as a hydrogen donator, and Thunberg's observations seem to show that it can so act, the loss of its labile hydrogen would convert it into pyruvic acid.

$$CH_3CHOH.\ COOH \longrightarrow CH_3.\ CO.\ COOH + H_2.$$

The hydrogen if not oxidized straightway will be transferred to some acceptor on its way to oxidation, whereas the first evolution of CO_2 from the original lactic acid molecule must involve a quite different type of change. It may, for instance, be evolved from pyruvic acid under the influence of a carboxylase—such catalysts being known to exist in animal tissues. The removal of hydrogen from saturated molecules may, in the cell, be a necessary preliminary to such a process as this, as well as making the carbon chain more open to the attack of molecular oxygen.

I now come to the main purpose of this lecture which is to describe an actual tissue constituent susceptible of autoxidation and able to play a part either as an acceptor or donator of hydrogen.

In 1888 de Rey-Pailhade[7] showed that yeast cells and aqueous extracts of yeast have the property of reducing sulphur to hydrogen-sulphide. Later he showed that many animal tissues possess the same property. Throughout a long series of communications upon the subject he has courageously maintained the view that the labile hydrogen thus shown to exist in living cells has important respiratory functions. His views as to the probable nature of the hypothetical substance which he supposed to carry this labile hydrogen and to which he gave the name *Philothion,* have been modified from time to time. In his latest writings he speaks of it as the hydride of a protein ("Hydrure d'albumine"). After the publications of Heffter, to which reference will immediately be made, he accepted the view that the labile hydrogen exists in sulphydryl groups, HS—.

[7] Comptes rendus, 1889.

In 1901 Mörner employed the delicate nitro-prusside reaction for the identification of cysteïn, and a little later Gola found by its use that a substance which presumably contains the HS— group, is characteristically present in proliferating plant tissues. Buffa then showed that the same color reaction is given by certain animal tissues.

In 1908 Heffter[8] applied the nitro-prusside test to a great number of tissues and tissue extracts and obtained positive results in many cases. Later, it would seem independently, V. Arnold[9] showed that the reaction under proper conditions is displayed by practically all organized animal tissues. He at first described it as a protein reaction, though recognizing that the plasma proteins gave a negative result.

A little later Arnold found that a strong nitro-prusside reaction may be given by protein-free extracts of tissues, and came to the conclusion that free cysteïn was the responsible substance. Although he did not isolate cysteïn, he considered that the evidence proved it to be a primary cell constituent in Kossel's sense.

Heffter, however, first gave definite form to the view that the presence of the HS— group, whatever its associations, may, owing to its labile hydrogen, be, in part at least, responsible for the reducing properties of protoplasm, and also, perhaps, indirectly for oxidations in the cell. Basing it upon known analogies he made the suggestion that during the autoxidation of the sulphydryl group pure oxide of hydrogen may be formed, and the peroxide oxygen then transferred, with or without the influence of a peroxidase, to other substances in the cell. If, further, HS— groups can be supposed to act continuously as promoters of cell oxidations, their own spontaneous oxidation to —S—S— groups must be reversible. In this connection Heffter called attention to the fact that cystine can be reduced to cysteïn by sodium sulphite, and suggested that in the cell some substance might, like the sulphite, act as an "acceptor" for the oxygen of the water molecule, leaving the hydrogen of this to reduce the di-sulphide group once more to sulphydryl groups. Heffter's views have become generally familiar owing to the publication of an admirable discussion by Thunberg,[10] in 1911, of the possibilities which underlie them.

Though very sugestive, these views have remained wholly theoretical. No experimental proof has been given of the existence of free cysteïn in the cell in the cells pictured by Arnold, nor has an HS— group been located in any other substance. Not without an isolation of the cell constituent actually responsible for the nitro-prusside reaction, and a study of its properties, could Heffter's theoretical views receive experimental support.

Such a substance I have recently succeeded in isolating and I propose to give you some account of it. To judge from color reactions it is present, I think, in all active animal tissues and probably in such vegetable cells as are engaged in the processes of growth and subdivision. It has so far been

[8] Heffter: Medicinisch-naturwissensch. Arch., 1908, I, 81.
[9] Arnold: Zeitsch. f. physiol. Chem., 1911, LXX, 300 and 314.
[10] Thunberg: Ergebnisse d. Physiol., 1911, XI, 328.

actually isolated from yeast, from striated muscle, and from mammalian liver alone. In them it is certainly responsible for the nitro-prusside reaction of the fresh tissue. The three cases are so diverse that it becomes highly probable that the color reaction as given by other tissues is due to the same substance.

It is present in very low concentration; perhaps to the extent of not more than 0.02 per cent of the fresh tissue. The significance of a tissue constituent is not, however, to be measured by the concentration in which it occurs. This is a point on which it is well to insist in connection with chemical studies of living tissues. A substance found in very small amounts may have a metabolic importance as great as that of another substance found in relatively large amounts. Many chemical reactions in the body form part of an ordered series. They are successive reactions. Now if one substance be changing into another through a series of intermediate products, then as soon as dynamic equilibrium has been established in the series—and to such equilibrium tissue processes always tend—the rate of production of any one intermediate product must be equal to the rate at which it changes into the next, and so throughout the series. But the rate of chemical change is measured by the product of the efficient concentration of the substance undergoing change and the velocity-constant of the change in question.

If K_1, K_2, K_3, K_4 are the respective velocity-constants of successive reactions in equilibrium, and [A], [B], [C], [D] are the corresponding concentrations of the substance we must find:

$$K_1[A] = K_2[B] = K_3[C] = K_4[D] = \text{etc.}$$

It follows that if K_1 is small and K_2 large, [A] must be large and [B] small and so on; clearly the significance of B is from any point of view no less than that of A. In the case, moreover, of the substance having catalytic functions, general experience leads to the expectation that it may be efficient as an agent though present in very low concentration. In certain measure such functions are attached to the substance to be now described.

You will not wish to be troubled with the technical details involved in the process of its isolation. It has shown itself to be a dipeptide containing cysteïn and glutamic acid. How the two amino-acids are linked in the molecule has not yet been determined with certainty but preliminary studies suggest that the dipeptide is cysteïnyl-glutamic acid:

$$HS\cdot CH_2\cdot CH\cdot NH_2\cdot CO-NH\cdot CH\cdot CH_2\cdot CH_2\cdot COOH.$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad COOH$$

I hope to convince you that the HS− group of the cysteïn component has dynamic functions within the cell. The reason that this group with its functions is found in a dipeptide of cysteïn rather than in free cysteïn itself is probably associated with the fact that for some reason the former is more resistant to the destructive changes of metabolism. The dipeptide is autoxidisable. When in neutral or slightly alkaline solution, it spontaneously oxidizes in the air at ordinary temperatures to form a compound with a disulphide linking on lines which are common to substances containing a sulphydryl group.

$$2(R-SH)+O = R-S-S-R+H_2O.$$

By various means it is possible to reduce the oxidized compound back to its original form. The HS− group of the dipeptide acts, one may say, as a hydrogen donator while the −S−S− group of its oxidized form acts as a hydrogen acceptor. The full significance of these properties only comes to light, however, when it is shown that the oxidation and reduction (or the removal and addition of hydrogen) are brought about in a reversible manner by factors present in the living cell itself. That fresh tissues possess a reduction potential such as to induce rapid reduction of the substance can be at once shown by the simplest technique. In one test-tube are placed a few cubic centimeters of distilled water and in another an equal amount of a weak solution of the dipeptide in its oxidized form, which gives of course no nitro-prusside reaction. A small weighed piece of fresh tissue (liver, kidney, muscle etc.) is then dropped into each. If the tubes be then placed in a bath at 35° C., it will be found that the fluid of that containing the dipeptide will, after an hour or two, give the nitro-prusside reaction with an intensity out of all proportion to the fluid of the control tube. The latter may give a slight reaction due to diffusion from the tissue of preformed reduced dipeptide. Under anaërobic conditions the change goes faster. Antiseptics may be used. The reduction may be followed in its stages by placing crystals of ammonium sulphate mixed with nitro-prusside on a tile. A couple of drops of the fluid followed by one of ammonia will show the increasing intensity of the colour reaction. It is difficult to obtain the reducing system in solution; but the dry powder, obtained by grinding a tissue with sand under alcohol, washing the residue with fresh alcohol on a filter, and allowing it to dry in the air, reduces efficiently. Such a powdered preparation in the case of liver, deprived as far as possible of connective tissue, has served well for quantitative observations.

It is next easy to show that conditions exist, on the other hand, in which factors present in tissues can promote oxidation of the reduced dipeptide. It is a circumstance familiar to all who have studied the reducing power of excised tissues, by the use, for instance, of methylene blue as an indicator, that the reduction potential rapidly falls off as survival processes progress. It must of course be remembered that conditions for reduction and oxidation vary with the substance concerned. In the case of the dipeptide survival changes involve a complete reversal of the existing relations. A tissue which has stood under aseptic conditions for a sufficient time fails to exhibit the nitro-prusside reaction, and it can be shown without difficulty that conditions then develop under which the reduced dipeptide is oxidized. As this oxidation goes on anaërobically some other substance must now act as a hydrogen acceptor. Especially upon actual autolysis does this change in the equilibrium of reducing and oxidizing agencies occur. If disintegrated tissues be suspended in four to five times their weight of chloroform water and allowed to auto-

lyse in corked flasks at 37°, the fluid during the first few hours will show a strong nitro-prusside reaction. This gradually fails in the fluid, and, ultimately, after a period varying with the particular tissue, also in the incompletely autolysed tissue fragments. The end point occurs soonest with kidney; more slowly with liver, because of its original high content of the reduced dipeptide; and still more slowly in the case of muscle. If when the color reaction has ceased a further quantity of reduced dipeptide be introduced so as to restore a strong reaction to the fluid, this will be found also to disappear on standing. The process goes on with undiminished velocity, and indeed, seemingly faster, under anaërobic conditions. That oxidation has occurred is shown by the fact that the process is easily reversible by reduction. If some of the fluid which has ceased to give the color reaction, and especially if its content of dipeptide had been increased on the lines mentioned, be boiled, filtered, and placed when cool over a piece of perfectly fresh tissue, an intense nitro-prusside reaction once more develops. The significance of this change of oxidation potential during survival and post-mortem changes need not be here discussed. For the moment I am concerned only to show that these exist in tissue mechanisms for both reduction and oxidation of the dipeptide. If, *in vitro*, the one has to disappear before the other can be displayed, it is yet perfectly probable that in the geographical organization of the cell both mechanisms are employed.

Considerations may now be put forward which indicate that the dipeptide does as a matter of fact play a real part in cell dynamics. Fresh tissues, of course, reduce methylene blue and so when itself in the reduced condition does the dipeptide. But as has just been shown, the former also reduces the latter. It would seem then that a mechanism in the tissues has a greater reduction (or lower oxidation) potential than the HS— group of the dipeptides. As a matter of fact, however, the relations depend upon the hydrogen-ion concentration of the medium. It should be remarked that the reduced condition of the dipeptide is more stable on the acid side of neutrality. The following facts are of interest.

If the substance in the oxidized (disulphide) form be added to a solution of methylene blue in contact with fresh tissue, then if the reaction of the system is even slightly on the acid side of neutrality, say at pH = 6.8, the reduction of the dye is greatly slowed, as the control preparation will at once show. The dipeptide acts here simply as a hydrogen acceptor and competes with the methylene blue in this respect, delaying or (according to its concentration) even preventing the reduction of the latter. If, however, the reaction of the medium be adjusted to pH 7.4, or made very slightly more alkaline than this, the relations change. The normal rate of reduction of methylene blue by the tissues used is then markedly *accelerated* by the addition of the *oxidized* dipeptide. The phenomena are best observed under anaërobic conditions and the observations should be made in test-tubes which can be evacuated or which are corked and so fitted with glass tubing that the air can be replaced by pure nitrogen. As the tissues contain their own supply of the substance the contrast between the behavior of a preparation to which dipeptide has been added and a control preparation is greater if the tissue used is first washed several times with sterilized distilled water. This removes a considerable part of the pre-existing dipeptide especially from the surface of the tissue. The tissue powder from alcohol, as described above, acts well and may also be washed before use.[11] Although the fact is not commented upon by authors who have used washed tissues in connection with methylene blue reduction, the concentration of the enzyme is reduced by extraction with water. In the experiments under discussion washing increases the contrast, but may increase the actual time required for reduction.

The following data will illustrate what has been said. In the experiments given the tissue preparation was in each case the liver of the rat, ground under alcohol, air-dried, and afterwards washed with distilled water. Similar results have been obtained, however, with other tissues washed and unwashed. The first comparison shows the reversal in the effect of added dipeptide when the reaction of the system passes from acid to neutral or slightly alkaline. The tubes were filled with nitrogen:

Tissue preparation	Oxidised dipeptide added	Me. blue 1:5000	Water	pH at beginning	Reduction time
0.5 gms.	0	0.5 c.c.	5 c.c.	7.5	3 hrs. 35 mins.
0.5 gms.	4 mgms.	0.5 c.c.	5 c.c.	7.5	1 hr. 30 mins.
0.5 gms.	4 mgms.	0.5 c.c.	5 c.c.	6.8	5 hrs. 20 mins.
0.5 gms.	8 mgms.	0.5 c.c.	5 c.c.	6.8	15 hrs. +

The following shows the acceleration of reduction due to the addition of oxidized dipeptide to the system in neutral or slightly alkaline conditions. The solution in each case was a phosphate buffer solution, the pH being adjusted after the addition of the dipeptide. Chloroform was added to all the tubes and there was a layer of toluol on the surface. The tubes were filled with nitrogen.

Tissue preparation	Buffer solution	Me. blue	Oxidised dipeptide added	pH	Reduction time
0.2 gms.	5 c.c.	0.5 c.c.	0	7.4	5 hrs. 45 mins.
0.2 gms.	5 c.c.	0.5 c.c.	4 mgms.	7.4	2 hrs. 40 mins.
0.2 gms.	5 c.c.	0.5 c.c.	0	7.8	3 hrs. 45 mins.
0.2 gms.	5 c.c.	0.5 c.c.	4 mgms.	7.8	1 hr. 40 mins.
0.5 gms.	5 c.c.	0.5 c.c.	0	7.8	2 hrs. 30 mins.
0.5 gms.	5 c.c.	0.5 c.c.	4 mgms.	7.8	0 hrs. 55 mins.
0.5 gms.	5 c.c.	0.5 c.c.	8 mgms.	7.8	0 hrs. 22 mins.

It seems clear that in the reactions described the —S—S— group of the dipeptides acts first of all as a hydrogen acceptor

[11] It is noteworthy that the tissues of small animals such as the rat show under all circumstances a greater reducing power than those of larger animals.

and under conditions of even slight acidity the resulting HS– groups are too stable to transfer the hydrogen to another acceptor. In neutral or slightly alkaline solutions, on the other hand, the hydrogen is transferred to the methylene blue. If this view be the right one, we have to recognize the important fact that the two reactions involved in the transference of hydrogen to the disulphide group under the influence of a tissue enzyme and its subsequent transference from sulphydryl groups to the methylene-blue acceptor, together run faster than the single reaction in which the dye is directly reduced by the tissue enzyme. The dipeptide then possesses what are essentially catalytic properties and could be fairly spoken of as a co-enzyme. Indeed, if tissues are very thoroughly washed, as in the observations of Thunberg referred to earlier, so that their power to reduce methylene blue is practically removed, the restoration of this power when the *oxidized* dipeptide is added at once gives the impression that it has the function of a co-ferment exerted on the lines suggested. But Thunberg's work suggests, of course, another possibility. He found, as already stated, that a number of substances such as succinic acid, malic acid and the like, including, as is noteworthy, glutamic acid, can under defined conditions restore reducing power to tissues deprived of it by washing. It is possible, therefore, that the dipeptide in the experiments just described was, when in neutral or alkaline solution, merely acting as a substance of this class. I am convinced, however, that this is not the case. The compounds which acted as hydrogen "donators" in Thunberg's experiments were undergoing an irreversible oxidation due to the removal of hydrogen atoms attached to carbon. I have evidence to show that this does not occur in the case of the dipeptide. Oxidation and reduction of this substance by tissue systems involve the thio group alone and the strictly reversible change:

$$-SH \quad HS- \rightleftarrows -S-S- + H_2.$$

The fact that the living cell actually contains a substance with the properties described gives strong support to the belief that the activation and transport of hydrogen represents one aspect of the chemical dyamics of tissues. It is probably an important aspect, though only a part of the manifold phenomena of biological oxidations. It would certainly be wrong to look upon it as more than partial. It is the duty of the bio-chemist to study as fully as possible every special line of chemical change within the living cell that he is fortunate enough to recognize and define. But he must remember that to relate each such line of change with the many others which accompany it—to study, that is, the organization of reactions in the cell—is a task which lies ahead.

STUDIES ON SOME OF THE NON-LIPOID COMPONENTS OF BLOOD SERUM IN RELATION TO ITS ANTIHEMOLYTIC PROPERTY

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In an earlier paper[1] it was reported that the protective power against hemolysis of guinea-pig corpuscles by sodium oleate and saponin is diminished in the serum from cases of pernicious anæmia and those in which the liver and spleen are implicated. Because of this finding it seemed worth while to study the substance or substances in the serum upon which the protective power against hemolysis depends. Direct experiments with lecithin and cholesterin, alone and in combination, did not yield evidence that either one is the sole substance in question.[2] The experiments undertaken, however, were not such that sweeping negative conclusions might be drawn in regard to the action of lecithin and cholesterin under different conditions. If lecithin and cholesterin are of importance in this connection, however, their action is very complex and is possibly dependent upon or influenced by other substances present in the serum. Before proceeding with further investigations along this line it appeared wise, therefore, to study other substances in the serum and to study the antihemolytic property of serum after various methods of treatment calculated to throw light on these points.

Many observations appear in the literature that are suggestive as to the substances other than the lipoids of serum which may be of importance in relation to its antihemolytic property. Joannovics and Pick[3] stated that hemolysis by oleic acid is inhibited by organ albumen. Fenyvessy[4] made the observation that hemolysis by soap solution is inhibited by albumen, but expressed the belief that some of this action is due to the calcium present. Liebermann[5] observed that serum albumen protected against hemolysis by soap and that similar protection is afforded by calcium solution. Lamar,[6] also, attributed to the action of serum proteins the protection afforded by serum against hemolysis of red blood cells and lysis of pneumococci by sodium oleate, and stated that the hemolytic action of soaps is due partly to their avidity for protein and not wholly to their ability to dissolve lipoids. Lamar[7] stated, however, that the removal of calcium from the serum did not alter the protective power exhibited by serum against hemolysis by soaps, but that the addition of boric acid to serum did diminish this protective power. Bayer[8] observed that the protective power of serum against hemolysis by bile salts was destroyed by digesting the serum with pepsin or trypsin, and suggested that the protective power of normal serum against this hemolysin was due to the serum albumen bodies. On the other hand, Sellards,[9] while studying the protective power of serum against hemolysis by bile and bile salts, found that precipitation of the proteins by heat did not affect this inhibitory property of the serum. W. W. Ford[10] reported that hemolysis by phallin, the hemolytic principle of a poisonous

mushroom (*Amanita phalloides*), was inhibited not only by blood serum but also by raw and boiled milk. The work of the observers mentioned, although not in entire accord, suggests therefore both albumen and calcium as possible factors in the protective power against hemolysis exhibited by blood serum. Evidence has also been presented to show that boric acid added to serum lowers its protective power against hemolysis by sodium oleate.

The experiments to be reported in this paper were undertaken to determine, if possible, what influence albumen, calcium and boric acid have on hemolysis by sodium oleate and saponin, and what relation these substances may have to the antihemolytic property of serum against these hemolytic agents. Similar experiments were carried out with dextrose and soluble starch.

To study these points quantitative measurements were made of the protective power of albumen, of calcium solution, and of the carbohydrate solutions against hemolysis by sodium oleate and saponin. Similar quantitative measurements were made of water and salt solution extracts of the dried residue of serum, of serum after boiling, after dialyzing, after digestion with trypsin, pepsin, and diastase, and after the addition of boric acid. The technique of making quantitative measurements of the protective power against hemolysis was the same as that reported in earlier papers on the protective power of human serum against hemolysis by sodium oleate,[1] and on studies of the possible rôle of lecithin and cholesterin in hemolytic processes.[2] A number of tubes, each containing 2 c. c. of a different dilution of hemolytic agent were set up in series and to each tube 0.25 c. c. of the material to be tested for protective power against hemolysis was added. This was heated at 37° C. in the water-bath for one-half hour, cooled for one-half hour, and then 0.25 c. c. of a 0.75 per cent suspension of washed guinea-pig cells was added as indicator. This preparation was incubated at 37° C. in the water-bath for two hours, allowed to sediment in the ice-box for one hour, after which a reading of hemolysis was made. The details of the technique are given in the earlier papers and will not be repeated here. In those titrations of protective power of serum against hemolysis, as in the ones in this paper, the tubes carried dilutions of sodium oleate from 1-45,000 to 1-100,000 in steps of 5000, and dilutions of saponin from 1-18,000 to 1-34,000 in steps of 2000. In such a series, 0.25 c. c. or normal serum diluted 1-20 protected against hemolysis by sodium oleate up to strengths of 1-50,000 and 1-55,000; and protected against hemolysis by saponin up to strengths of 1-18,000 and 1-20,000.

I. Studies with Proteins

The properties of proteins in relation to hemolysis by sodium oleate and saponin were studied by several methods.

(a) The protective power of serum against hemolysis was determined after

(1) Boiling and
(2) Boiling and filtering.

(b) The protective power of serum against hemolysis was determined after digestion with

(1) Pepsin.
(2) Trypsin.

(c) The protective power against hemolysis of crystallized egg albumen dissolved in salt solution was determined.

(d) The protective power of dialyzed serum was determined both before and after the precipitated globulins had been removed by filtration.

(a) For the studies of boiled serum, normal serum was prepared for titration in the manner already detailed in a previous paper. The blood was taken by veni-puncture into a clean, dry syringe, expelled into a clean, dry test-tube, allowed to clot, and within one hour after the veni-puncture the serum was removed from the clot and immediately inactivated in the water-bath at 56° C. for one hour. This was kept in the ice-box till just before use—always within twenty-four hours from the time of veni-puncture—and then diluted 1-20 with normal salt solution. The serum thus prepared was divided into four portions. One portion was boiled vigorously for five minutes; another portion was boiled the same way and filtered through two layers of filter paper and kieselguhr; and another portion was similarly filtered but not boiled. With these three specimens and with the fourth which was neither filtered nor boiled titrations for protective power against hemolysis by sodium oleate and saponin were carried out. They resulted as follows:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-50,000.
Boiled serum—partial hemolysis up to and including dilution 1-65,000.
Boiled serum, filtered—partial hemolysis up to and including dilution 1-65,000.
Filtered serum—partial hemolysis up to and including dilution 1-50,000.

Saponin

Normal serum—partial hemolysis up to and including dilution 1-20,000.
Boiled serum—partial hemolysis up to and including dilution 1-28,000.
Boiled serum, filtered—partial hemolysis up to and including dilution 1-28,000.
Filtered serum—partial hemolysis up to and including dilution 1-20,000.

These results show that the boiling of serum lowers its protective power against hemolysis by sodium oleate and saponin. This diminution is not increased by filtration of the boiled serum.

(b) For studies of the action of ferments on the protective power of serum against hemolysis, pepsin was added to 10 c. c. of normal serum prepared as above and trypsin added to another 10 c. c. portion. These preparations were incubated at 37° C. over night, then filtered through kieselguhr till clear, and inactivated at 56° C. in the water-bath for one hour. Normal serum not treated with ferments was similarly incubated, filtered and inactivated at the same time. Titrations with these preparations and of normal untreated serum for

protective power against hemolysis by sodium oleate and saponin resulted as follows:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Serum digested with trypsin—complete hemolysis up to and including dilution 1-90,000 plus.
(This hemolysis was complete at the end of an hour's incubation. No doubt it would have gone far above 1-90,000 had the dilutions been carried further.)
Serum digested with pepsin—partial hemolysis up to and including dilution 1-90,000.
Serum incubated and filtered—partial hemolysis up to and including dilution 1-55,000.

Saponin

Normal serum—partial hemolysis up to and including dilution 1-18,000.
Serum digested with trypsin—complete hemolysis up to and including dilution 1-30,000 plus.
(This hemolysis was complete at the end of half an hour's incubation. The hemolysis would have been seen in dilutions much higher than 1-30,000 if the titration had been carried further.)
Serum digested with pepsin—partial hemolysis up to and including dilution 1-26,000.
Serum incubated and filtered—partial hemolysis up to and including dilution 1-18,000.

The results obtained in these experiments show that, if serum is digested with trypsin or pepsin, the protective power againt hemolysis by sodium oleate and saponin is markedly diminished. When serum is digested with trypsin the reduction in protective power is so severe that it almost amounts to a complete destruction of this property.

The reduction in protective power shown in these experiments might be due to several factors. That it is due to the action of the ferments on the serum is shown by control experiments carried out to establish this point:

(1) Serum was treated with ferments that had been killed and under these conditions there was no diminution in its antihemolytic power.

(2) Ferments were added to salt solution, the salt solution was incubated, inactivated, and filtered as was done with serum. The preparation showed no hemolytic activity.

(3) Serum treated with live ferments and then inactivated showed no hemolytic properties.

(c) To study the influence of crystallized egg albumen on hemolysis by sodium oleate and saponin, albumen crystals prepared by Dr. V. R. Mason according to the method of Hopkins and Pinkus[11] were used. Of these egg albumen crystals, 70 milligrams were dissolved in 10 c. c. of distilled water. This was divided into two 5 c. c. portions and one portion filtered five times through filter paper till water clear, to remove any trace of globulin present. To each of these 5 c. c. portions 45 milligrams of sodium chloride were added, resulting in a solution of albumen in 0.9 per cent salt solution containing about twice as much albumen as normal serum after it has been diluted 1-20 as used in our titrations for protective power against hemolysis. The solution of crystallized egg albumen was used in the titration as serum diluted 1-20, that is, 0.25 c. c. was put in each tube of the series, so that in each tube there was twice as much egg albumen as there was serum albumen in each tube when serum was used. Titrations of the protective power against hemolysis were made with each of the solutions of crystallized egg albumen prepared as above and these compared with a hemolytic series set up with guinea-pig corpuscles unprotected by serum or other substance. In this last hemolytic series the usual volume, 2.5 c. c. in each tube of the hemolytic series, was made up by the addition of 0.25 c. c. of sterile normal salt solution instead of 0.25 c. c. of serum diluted 1-20. The results obtained were as follows:

Sodium Oleate

Unprotected guinea-pig corpuscles—partial hemolysis up to and including dilution 1-160,000.
Albumen solution, filtered—complete hemolysis up to and including dilution 1-170,000 plus.
Albumen solution, unfiltered—complete hemolysis up to and including dilution 1-170,000 plus.
(In these last two titrations hemolysis would probably have gone further but the series was not carried to dilutions greater than 1-170,000.)

Saponin

Unprotected guinea-pig corpuscles—partial hemolysis up to and including dilution 1-80,000.
Albumen solution, filtered—partial hemolysis up to and including dilution 1-80,000.
Albumen solution, unfiltered—partial hemolysis up to and including dilution 1-80,000.

These experiments demonstrate that crystallized egg albumen dissolved in normal salt solution, used in amounts twice as great as serum albumen when serum is used in a titration for protective power against hemolysis, does not afford protection against hemolysis by sodium oleate and saponin. On the contrary, the presence of egg albumen seems to increase the hemolytic power of sodium oleate.

(d) In studying the relation of the electrolytes of serum to hemolysis by sodium oleate and saponin, some normal serum was dialyzed and this serum was tested for protective power againt hemolysis. Some of the dialyzed serum was filtered to remove the precipitated globulins and this globulin-free, or at least globulin-poor, serum also was tested for protective power against hemolysis. This experiment will be given in detail below; but it is interesting to observe here that the diminution of protective power which is found to result from the dialysis of serum is increased a little, but only to a negligible degree, by removal of the precipitated globulins by filtration.

II. Studies with Calcium

To determine, if possible, what rôle calcium salts play in the protective power exhibited by serum against hemolysis by sodium oleate and saponin, several different experiments were carried out.

(a) Serum was treated with potassium oxalate and the protective power of the serum thus treated was tested.

(b) Calcium chloride was added to serum and this serum was titrated for protective power against hemolysis.

(c) Solutions of calcium chloride in normal salt solution were tested for protective power against hemolysis.

(a) To determine the effect on protective power against hemolysis when any available calcium in the serum is rendered inert as far as possible by treating it with potassium oxalate, 500 milligrams of potassium oxalate crystals were added to 0.5 c. c. of serum. This was allowed to stand for thirty minutes and was then diluted to 10 c. c. with sterile normal salt solution and kept in the incubator at 37° C. over night. The next morning just before use the crystals remaining were thoroughly sedimented by centrifugation. Titrations carried out with this oxalated serum diluted 1-20 resulted as follows:

SODIUM OLEATE

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Oxalated serum—partial hemolysis up to and including dilution 1-65,000.

SAPONIN

Normal serum—partial hemolysis up to and including dilution 1-20,000.
Oxalated serum—partial hemolysis up to and including dilution 1-20,000.

These experiments show that, if serum is oxalated, its protective power against hemolysis by sodium oleate is diminished slightly. They also show that this treatment does not lower the protective power against hemolysis by saponin.

(b) The experiment detailed above shows that oxalated serum has lost some of its protective power aginst hemolysis by sodium oleate as compared with normal serum. It seemed worth while, therefore, to add calcium chloride to serum to see if thereby its protective power against hemolysis would be increased. One specimen of serum was diluted 1-40 with a solution of calcium chloride in normal salt solution. In this solution there were 62 milligrams of calcium chloride per 100 c. c. of normal salt solution. The preparation resulting was serum diluted 1-40 (or one-half the strength as ordinarily used) in the presence of a great excess of calcium chloride, as compared with a similar quantity of whole normal blood. Another portion of the same serum was diluted 1-40 with sterile normal salt solution. Titrations carried out with these preparations and with the same serum diluted 1-20 as in the ordinary technique resulted as follows:

SODIUM OLEATE

Normal serum (1-20)—partial hemolysis up to and including dilution 1-55,000.
Normal serum (1-40)—partial hemolysis up to and including dilution 1-85,000.
Normal serum (diluted 1-40 with normal salt solution containing 62 milligrams of calcium chloride per 100 c. c.)—partial hemolysis up to and including dilution 1-70,000.

SAPONIN

Normal serum (1-20)—partial hemolysis up to and including dilution 1-20,000.
Normal serum (1-40)—partial hemolysis up to and including dilution 1-24,000.
Normal serum (diluted 1-40 with normal salt solution containing 62 milligrams of calcium chloride per 100 c. c.)—partial hemolysis up to and including dilution 1-24,000.

These experiments show that calcium chloride, when added to serum in very small amounts, but relatively in great excess as compared with that present in normal blood serum when diluted 1-20 for use, is capable of increasing the protective power of serum slightly against hemolysis by sodium oleate. This same procedure does not increase the protective power of serum against hemolysis by saponin.

(c) In order to study further the possible rôle of calcium in the blood in relation to the protective power against hemolysis exhibited by serum, 62 milligrams of calcium chloride were dissolved in 100 c. c. of normal salt solution and this preparation was tested for protective power against hemolysis. It was used just as normal serum diluted 1-20, *i. e.*, 0.25 c. c. of the solution of calcium chloride in normal salt solution was added to each tube of the hemolytic series. Since the calcium solution contained much more calcium than serum, and was used without dilution, there were many more times as much calcium in each tube of the hemolytic series as when serum is used in the ordinary titration. The readings made were as follows:

SODIUM OLEATE

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Calcium in salt—complete hemolysis up to and including dilution 1-90,000.
(The hemolysis would probably have gone higher than 1-90,000, but the titration was not carried further.)

SAPONIN

Normal serum—partial hemolysis up to and including dilution 1-20,000.
Calcium in salt—complete hemolysis up to and including dilution 1-30,000.
(The hemolysis up to 1-30,000 was complete almost immediately and would probably have gone further had the titration been carried on to greater dilutions.)

These experiments show that calcium chloride in salt solution, present in much greater concentration than when serum is used, affords little, if any, protection against hemolysis by sodium oleate. The calcium chloride solution does, however, slow the reaction somewhat with sodium oleate, for at the end of 1½ hours' incubation hemolysis had proceeded only to dilution 1-70,000, whereas, in all the titrations set up with unprotected guinea-pig corpuscles previously recorded, hemolysis at that time had always proceeded to dilutions above 1-100,000 and sometimes as high as dilutions of 1-150,000. Such a calcium chloride solution does not protect against hemolysis by saponin.

III. STUDIES WITH CARBOHYDRATES

There is nothing to suggest sugar as a substance in the serum of importance in relation to protective power against hemolysis, and we have no reason to expect that sugar can inactivate a hemolytic agent. Evidence has been presented, however, to show that a medium containing sugar will preserve red blood cells better than a similar solution without sugar. Carbohydrates might, therefore, be of great importance in hemolysis, if not as a substance neutralizing the hemolytic agent, at least, perhaps, rendering the red blood

cells less subject to its disintegrating influence. Furthermore, carbohydrates, as important constituents of normal blood, are worthy of some study in relation to their protective power against hemolysis. This point was investigated by several methods:

(a) Quantitative measurements were made of the antihemolytic power of a solution of dextrose and of soluble starch in normal salt solution.

(b) Dextrose and soluble starch were added to serum, and quantitative measurements were made of the antihemolytic power of serum thus treated.

(c) Serum after digestion with diastase was tested for protective power against hemolysis.

(a) One hundred milligrams of pure dextrose were dissolved in 100 c. c. of sterile normal salt solution. The salt solution then contained the same concentration of sugar as normal blood. It was further diluted 1-5, and 0.25 c. c. of this preparation was put in each tube of the hemolytic series so that the sugar was four times as strong as in the serum diluted 1-20 used in the ordinary test. A similar preparation with equal quantities of soluble starch * was prepared and used in the same way. The titrations carried out resulted as follows:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Dextrose in salt solution—complete hemolysis up to and including dilution 1-90,000.
Soluble starch in salt solution—complete hemolysis up to and including dilution 1-90,000.
(The hemolysis in the last two tests was complete after half an hour's incubation. It would have gone far above 1-90,000 had the dilutions been carried further.)

Saponin

Normal serum—partial hemolysis up to and including dilution 1-18,000.
Dextrose in salt solution—complete hemolysis up to and including dilution 1-30,000.
Soluble starch in salt solution—complete hemolysis up to and including dilution 1-30,000.
(The hemolysis in the last two titrations was complete after half an hour's incubation. It would have gone far above 1-30,000 had the dilutions been carried further.)

These experiments show that neither dextrose nor soluble starch dissolved in salt solution protects against hemolysis by sodium oleate or saponin.

(b) Normal serum was inactivated as usual and 10 milligrams of dextrose were added to one 5 c. c. portion and 10 milligrams of soluble starch to another. The carbohydrates dissolved readily in the serum. These preparations were kept in the ice-box over night. Dilution of 1-20 was carried out as usual just before use and titration for any protective power of these sera and a normal serum prepared in the usual way yielded the following results:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Normal serum plus soluble starch—partial hemolysis up to and including dilution 1-50,000.
Normal serum plus soluble starch—partial hemolysis up to and including dilution 1-55,000.

Saponin

Normal serum—partial hemolysis up to and including dilution 1-18,000.
Normal serum plus dextrose—partial hemolysis up to and including dilution 1-20,000.
Normal serum plus soluble starch—partial hemolysis up to and including dilution 1-20,000.

These experiments show that serum to which dextrose or soluble starch has been added, even in relatively large quantities (200 milligrams of carbohydrate per 100 c. c. serum) shows no appreciable alteration in protective power against hemolysis by sodium oleate and saponin. The titrations recorded are within the limits of normal with the technique used.

(c) Diastase was added to 10 c. c. of normal serum and this solution was incubated for eighteen hours. It was then filtered twice through kieselguhr and inactivated in the water-bath at 56° C. for one hour. Some serum from the same source not treated with diastase was similarly incubated, filtered, and inactivated. Dilution of 1-20 was carried out as usual and titration for the protective power of these sera and of a normal serum prepared in the usual way yielded the following results:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Normal serum (incubated and filtered but not treated with any ferment)—partial hemolysis up to and including dilution 1-55,000.
Normal serum after incubation with diastase—complete hemolysis up to and including dilution 1-90,000 (the hemolysis in dilutions as far as carried 1-90,000 was complete after one hour's incubation).

Saponin

Normal serum—partial hemolysis up to and including dilution 1-18,000.
Normal serum (incubated and filtered but not treated with any ferment)—partial hemolysis up to and including dilution 1-18,000.
Normal serum after incubation with diastase—complete hemolysis up to and including dilution 1-30,000 (the hemolysis was complete in all the dilutions set up after one-half hour's incubation).

These experiments demonstrate that, when serum is digested with diastase, the protective power against hemolysis by sodium oleate and saponin is markedly diminished, if not completely destroyed. This diminution in protective power is due to the action of the ferment on the serum as shown by control experiments carried out with the diastase as with trypsin and pepsin already detailed:

(1) Serum treated with inactivated ferment did not show a diminution in antihemolytic property.

(2) Salt solution treated with diastase showed no hemolytic activity.

* The soluble starch used was obtained from the Department of Physiological Chemistry of The Johns Hopkins Medical School through the kindness of Professor Walter Jones. It was a specimen made from compressed yeast in an attempt to get yeast nucleic acid by the method of Clark and Schriver.

(3) Serum treated with live ferments and then inactivated showed no hemolytic activity.

IV. Miscellaneous Studies

In addition to studies with lecithin and cholesterin[2] and with proteins, calcium, and carbohydrates, certain other investigations were carried out in a search for the substance or substances in the serum responsible for the protective power exhibited by it against hemolysis by sodium oleate and saponin.

(a) The dried residue of serum taken up in distilled water and in sterile normal salt solution was tested for protective power against hemolysis.

(b) The protective power of serum after dialysis was measured quantitatively.

(c) Quantitative measurements of the protective power of serum after the addition of boric acid were made; and of boric acid dissolved in normal salt solution.

(a) To determine whether any protective substance against hemolysis by sodium oleate and saponin could be demonstrated in the dried residue of serum, several methods of drying the serum were tried, and that found most satisfactory and apparently least likely to introduce unknown factors was drying in the air. Serum in accurately measured 2 c. c. portions was put on chemically clean, sterile watch crystals and these, protected from dust as much as possible, were dried for twenty-four hours in front of an electric fan. This dried residue was pulverized very thoroughly and that derived from each 2 c. c. portion of serum was put in a separate test tube. Ten cubic centimeters of sterile distilled water were added to two of the tubes containing the pulverized residue from 2 c. c. serum, and ten cubic centimeters of sterile normal salt solution were added to two others. One of these to which water and one to which normal salt solution had been added, were boiled gently for five minutes. This resulted in four preparations:

(1) Dried residue of serum in cold water.
(2) Dried residue of serum boiled in water.
(3) Dried residue of serum in cold normal salt solution.
(4) Dried residue of serum boiled in normal salt solution.

When the water or salt solution was added to the powdered serum residue and shaken, the preparations took on a cloudy, brownish appearance. They were put in the incubator at 37° C. over night and when looked at in the morning no sedimentation had occurred in the tubes that had been boiled and practically none in the others. Attempts made to clear the solutions by centrifugation were unsuccessful. A portion of each preparation filtered twice through ordinary filter paper did not become clear. Accordingly, titrations for any protective power against hemolysis were caried out with these suspensions (rather than extracts) of the whole dried residue of serum, using them exactly as serum diluted 1-20; *i. e.*, 0.25 c. c. was put in each tube of the hemolytic series. In these preparations the serum components were present in four times the strength that they were in normal serum as used in a titration. Constant quantitative results could not be obtained on repetition of these experiments, but the gross results were such that definite conclusions might be drawn. For example the titrations of one day resulted as follows:

Sodium Oleate

Serum residue in cold water—no hemolysis in dilution 1-50,000.
Serum residue boiled in water—no hemolysis in dilution 1-50,000.
Serum residue in cold normal salt solution—hemolysis in dilution 1-50,000, but not in 1-55,000.
Serum residue boiled in normal salt solution—hemolysis in dilution 1-50,000, but not in dilution 1-55,000.

Saponin

Serum residue in cold water—no hemolysis in dilution 1-18,000.
Serum residue boiled in water—no hemolysis in dilution 1-18,000.
Serum residue in cold normal salt solution—no hemolysis in dilution 1-18,000.
Serum residue boiled in normal salt solution—no hemolysis in dilution 1-18,000.

Other experiments carried out in the same way did not duplicate these figures exactly, but demonstrated, as do these, that such a preparation of the dried residue of serum has marked protective power against hemolysis by sodium oleate and saponin.

(b) To determine what influence the removal of electrolytes from serum would have on its protective power against hemolysis by sodium oleate and saponin, normal serum was subjected to dialysis and the protective power of such serum measured quantitatively. Normal serum in 5 c. c. quantities was accurately measured into collodion sacks and dialysis carried on in running tap-water for twenty-four hours. The contents of the sack were then put into a chemically clean, sterile container. The dialyzing sack was washed out repeatedly with small amounts of sterile, normal salt solution and these washings added to the other fluid from the sack. Another similar preparation was filtered through kieselguhr till clear to remove the precipitated globulins before the addition of enough salt solution to render them soluble. Each of these preparations was brought up to a total volume of 100 c. c. by the addition of sterile normal salt solution, resulting in a dilution of 1-20 of the 5 c. c. serum originally introduced into the dialyzing sack. This dialyzed serum, one portion filtered before the addition of much salt solution, and another not filtered at all, diluted 1-20 in this manner, was used in a titration for protective power against hemolysis exactly as normal serum diluted 1-20 in an ordinary titration. The readings made were as follows:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-55,000.
Dialyzed serum—partial hemolysis up to and including dilution 1-80,000.
Dialyzed serum filtered—partial hemolysis up to and including dilution 1-85,000.

Saponin

Normal serum—partial hemolysis up to and including dilution 1-20,000.
Dialyzed serum—partial hemolysis up to and including dilution 1-22,000.
Dialyzed serum filtered—partial hemolysis up to and including dilution 1-30,000.

These experiments show that dialysis of serum lowers the protective power manifested by it against hemolysis by sodium oleate and saponin; and that this diminution of protective power is increased by removal of the precipitated globulins by filtration. In the repetition of the experiments the exact figures given above could not be duplicated, but all were such that the same conclusions were unavoidable. One cannot, however, draw conclusions from them as to the quantitative diminution of protective power against hemolysis after dialysis of serum.

(c) Liebermann and Fenyvessy [6] made the observation that the protective power of serum against soap hemolysis was inhibited by adding boric acid to the serum; and this finding was confirmed by Lamar.[7] In this study it was thought important to test out this property of boric acid in relation to hemolysis by sodium oleate and saponin. Accordingly, quantitative measurements were made of the protective power against hemolysis of:

(a) Normal serum to which boric acid had been added.

(b) A solution of boric acid in salt solution.

(a) Boric acid crystals were added in excess to 0.5 c. c. of serum and this was diluted to 10 c. c. (1-20) with sterile normal salt solution and allowed to stand in the incubator over night. The next morning the excess boric acid crystals were removed by centrifugation. As in the usual titrations 0.25 c. c. of this serum was added to each of the tubes in the hemolytic series. The readings made were as follows:

Sodium Oleate

Normal serum—partial hemolysis up to and including dilution 1-55,000.

Normal serum to which boric acid had been added—partial hemolysis up to and including dilution 1-90,000 (the hemolysis probably would have proceeded further but the titration was not carried to higher dilutions).

Saponin

Normal serum—partial hemolysis up to and including dilution 1-20,000.

Normal serum to which boric acid had been added—partial hemolysis up to and including dilution 1-20,000.

These experiments show that boric acid added to serum markedly reduces the protective power of the serum against hemolysis by sodium oleate, but does not diminish the protective power against hemolysis by saponin.

The control tubes set up with each titration [8] showed that the serum to which boric acid had been added was not in itself hemolytic.

(b) Boric acid crystals in excess were added to sterile salt solution and this was allowed to remain in the incubator over night. Just before use the next morning, the excess boric acid crystals were removed by centrifugation and 0.25 c. c. of the clear solution was added to each tube of the hemolytic series. At the end of one and one-half hour's incubation the hemolysis was complete as far as the titrations were carried; *i. e.*, 1-90,000 for sodium oleate, and 1-30,000 for saponin. This experiment indicates that boric acid in itself has no protective power against hemolysis. It further showed that boric acid has little if any power to cause hemolysis, for under the conditions of the experiment the addition of even a weak hemolytic agent, instead of the salt solution to which boric acid had been added, would have caused complete hemolysis almost immediately in all dilutions prepared, instead of after one and one-half hour's incubation.

Summary of Findings

(I) The boiling of serum lowered slightly its protective power against hemolysis by sodium oleate and saponin. The protective power of serum was very markedly diminished also by digestion of the serum by trypsin and pepsin. Egg albumen did not exhibit protective power against hemolysis by these agents.

(II) Serum treated with potassium oxalate showed a slight diminution in protective power against hemolysis by sodium oleate, but no reduction in protection against hemolysis by saponin. Calcium chloride added to serum increased slightly the protective power against hemolysis by sodium oleate, but not by saponin. Calcium chloride in salt solution exhibited no protective power against hemolysis by sodium oleate or saponin, but did apparently slow the progress of the hemolytic process with sodium oleate.

(III) Dextrose, or soluble starch dissolved in salt solution had no protective power against hemolysis by sodium oleate or saponin. Serum digested with diastase, however, showed a marked reduction in protective power against both sodium oleate and saponin.

(IV) (a) The dried residue of serum when suspended in distilled water or normal salt solution exhibited marked protective power against hemolysis by both sodium oleate and saponin.

(b) Serum when dialyzed exhibited a marked diminution in protective power against hemolysis by both sodium oleate and saponin. When the dialyzed serum was filtered to remove the precipitated globulins, the diminution in protective power was increased.

(c) Serum treated with boric acid showed a diminution in protective power against hemolysis by sodium oleate, but no diminution in protection against saponin hemolysis. Boric acid in salt solution had no protective power against either sodium oleate or saponin hemolysis.

It is very difficult to draw conclusions from the experiments discussed above. The fact that when serum is boiled, thereby coagulating the albumen, the protective power against hemolysis by both hemolytic agents is diminished indicates that this property of the serum is in some way dependent upon the albumen present. The diminution in protective power resulting from digestion of the serum by pepsin and trypsin lends further support to this supposition. If this is correct, however, it is some property peculiar to albumen found in serum or perhaps closely allied proteins, or a property dependent upon the physical state in which the albumen is held in the serum, since crystallized egg albumen dissolved in normal salt solution exhibits no protective power against hemolysis by the hemolytic agents used. The fact that crystallized albu-

men has no protective power also suggests that the protective power of the serum may be due to the globulin fraction. When the globulins of the serum were precipitated by dialysis and removed by filtration, it was seen that there was a slight diminution in protective power as compared with that afforded by serum dialyzed but not filtered. This would indicate that the globulin fraction is of some importance, but the difference between dialyzed serum from which the globulin had been largely removed and dialyzed serum from which the globulin had not been removed was so slight that no conclusions may be drawn from it, except that probably the globulin had little if anything to do with the inhibition of hemolysis by sodium oleate and saponin. It must also be kept in mind that the diminution in protective power of serum after boiling, after digestion, and after dialysis and filtration is possibly due in part at least to changes in the serum other than those produced in the protein fraction. So, although these experiments are strongly suggestive that the albumen of the serum, or at least the physical state in which it is held, is an important factor in the inhibition by serum of hemolysis by sodium oleate and saponin, they are not conclusive.

The experiments with calcium salts indicated definitely that calcium affords no protection against saponin hemolysis. The protection exhibited by calcium salts against hemolysis by sodium oleate was very slight, even though the calcium salts were present in more than twenty times the strength normally occurring in the serum as used. This can probably be explained by the fact that insoluble calcium oleate was formed from part of the sodium oleate present. These results seem to justify the conclusion that the calcium in serum is not responsible for the protective power manifested by it against hemolysis by sodium oleate and saponin, and that, if it plays any rôle at all in this protective power, it is indirect.

The finding that serum digested with diastase shows marked diminution in protective power against both hemolytic agents used, suggested the possibility that the carbohydrates of the serum were important factors in the phenomena under consideration. But when it was found that neither dextrose nor soluble starch exhibited protective power against hemolysis by these hemolysins, and that none of these substances added to serum increased its antihemolytic power, the conclusion was unavoidable that the diminution in protective power was not dependent upon changes in the carbohydrates of the serum by the action of diastase. It seems more probable that the diminution in protective power was due to other changes taking place in the serum as a result of the diastatic action.

The protective power of the dried residue of serum suspended in normal salt solution and in distilled water showed that this property of the serum is not destroyed by drying. The diminution of the protective power of serum resulting from removal of the electrolytes by dialysis indicates that these are of importance, but the negative findings, in direct experiments with calcium salts alone, do not support this hypothesis, at least as far as calcium salts are concerned. Again, it is probable that it is not merely the removal of the electrolytes but other changes in the serum incident to this procedure which are responsible for the resulting diminution in protective power.

It appears almost certain that the protection by serum against hemolytic agents is a colloidal phenomenon and that whatever the substance in the serum may be which exerts this action, the physical state in which it is held in the serum is important. It is possible, therefore, that many of the procedures carried out in these experiments which altered the colloidal state of the serum were, by reason of this action, responsible for the changes in protective value of the serum, rather than any effect they had on any one particular substance in the serum. Boiling and drying serum, the changes in reaction incident to digestion of serum by trypsin, pepsin and diastase, and removal of electrolytes, so altered the physical properties of the serum that if the physical state in which the protective substance is held in the serum is important, no further explanation for the reduction in protective power need be sought. Indeed the reduction in protective power of serum resulting from such widely different procedures, leads to the conclusion that the physical state of the serum is important and that the protection afforded by serum against hemolysis by certain hemolytic agents is a very complex process.

Conclusions

(1) Treatment of serum which alters the physical state or removes all or a part of the proteins diminishes the protective power of the serum against hemolysis by sodium oleate and saponin.

(2) Calcium alone is not the substance in serum upon which the protective power of the serum against hemolysis by sodium oleate and saponin depends.

(3) Dextrose or soluble starch have no power to protect against hemolysis by sodium oleate and saponin *in vitro* and probably, therefore, carbohydrates exert no such influence in the blood.

(4) Boric acid added to serum diminishes the protective power against hemolysis by sodium oleate, but does not influence the protection against hemolysis by saponin.

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THE NUCLEUS IN THE HUMAN RESTIFORM BODY[1]

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Introduction

While studying the human brain I came across a small nucleus in the restiform body, which, so far as I can learn, has not been described in the literature. It occurred to me that this nucleus was probably constant in the human restiform body, and as a result, I undertook the examination of quite a number of human brains. I was doubtful at first whether the nucleus, which I am about to describe, could always be demonstrated, but thorough examination revealed its presence in the restiform body of all the human brains that I examined. The results of these studies follow.

Report of Cases

All brains were fixed in formaldehyde solution and cut transversely in serial sections, each 25 microns thick, from the medulla oblongata and pons; the sections were then stained by the Nissl method and microscopic studies made. The observations have been tabulated and figured as follows:

In addition to the tabulated cases, the brains of one adult and one fetus, stained by the Nissl method, and those of three adults, stained by the Weigert method, were examined. In every one of these cases there was found in the restiform body a small nucleus, occupying a position similar to that described in the previous cases. None shows the hilum of the first case, although in some there is evidence of a slight curvature in transverse sections, suggesting a very incomplete hilum in the nucleus. The long axis of the nucleus, when it appears as an elongated mass, in transverse sections, always runs in a dorso-medial-ventro-lateral direction. In the Weigert preparations, a dense mass of nerve fibers is seen streaming to and from the hilum and also surrounding it. However, there is no evidence as to the origin of these nerve fibers.

The Weigert studies of the cells found in the nucleus are omitted in this paper, since this method of study does not lend itself to our present purpose. The fetus stained by the Nissl method is not described in detail here, because some of the

Case	Upper and lower levels	Position in restiform body	Development of nucleus	Form of nucleus	Longitudinal diameter	Size of cells in nucleus	Shape of cells in nucleus	Hilum
I.	First comes into view at the level where the inferior olive is marked and the superior vago-glosso-pharyngeal roots are still seen. Disappears where the inferior portion of the accessory auditory nucleus appears.	Appears as a small mass of gray matter in the dorso-medial part of the restiform body (see Fig. 1).	Fully developed.	⊃-shaped (see Fig. 5).	Distance between inferior and superior poles, 1.3 mm.	11.1–22.2 μ..	Round or fusiform.	Pronounced.
II.	Same.....	Same.....	Poor.....	Round or elliptical. (Fig. 6.)	0.6 mm.....	7.8–18.5 μ...	Same.....	Absent or imperfect.
III.	Same.....	Same.....	Poor.....	Elongated with constriction in middle. (Fig. 7.)	0.9 mm.....	7.4–22.2 μ...	Same.....	.....
IV.	Same.....	Same.....	Traces only...	Round. (Fig. 8.)	0.6 mm.....	11.1–18.5 μ..	Same.....	Absent.
V.	Same.....	Same.....	Traces only...	Round. (Fig. 9.)	0.5 mm.....	11.1–25.9 μ..	Same.....	Absent.
VI.	Same.....	Same.....	Poor.....	Round. (Fig. 10.)	0.6 mm.....	11.1–22.2 μ..	Same.....	Absent.
VII.	Same.....	Same.....	Not advanced.	Round. (Fig. 11.)	0.8 mm.....	11.1–25.9 μ..	Same.....	Incomplete.
VIII.	Same.....	Same.....	Not advanced.	Round. (Fig. 12.)	0.6 mm.....	11.1–18.5 μ..	Same.....	Incomplete.

[1] Read before the thirty-first annual meeting of the Okayama Medical Association and the nineteenth annual meeting of the Japanese Neurological Association.

Fig. 1

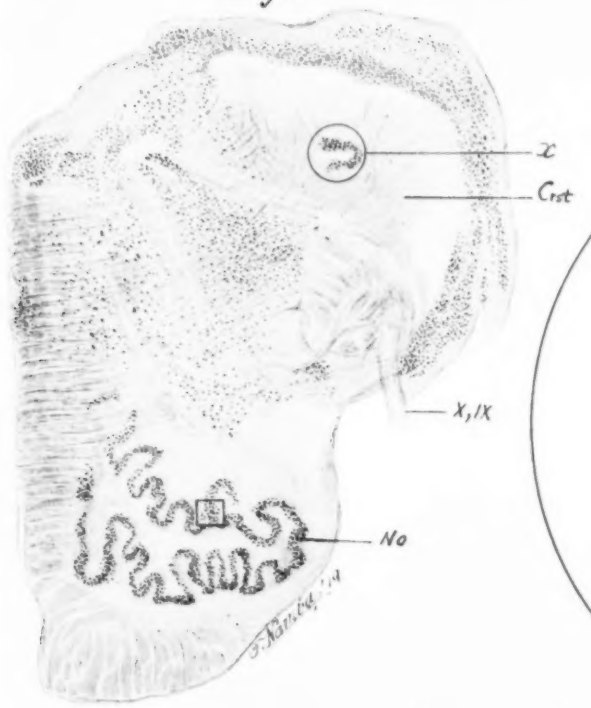

Fig. 2

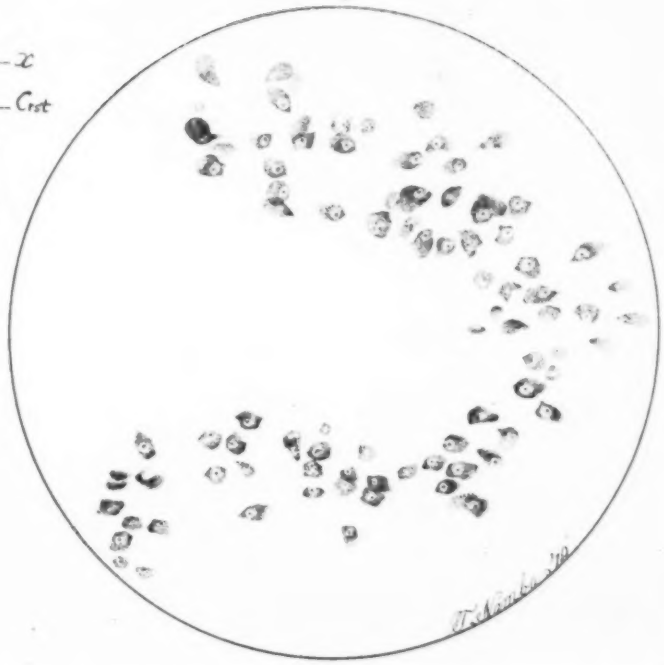

Fig. 3

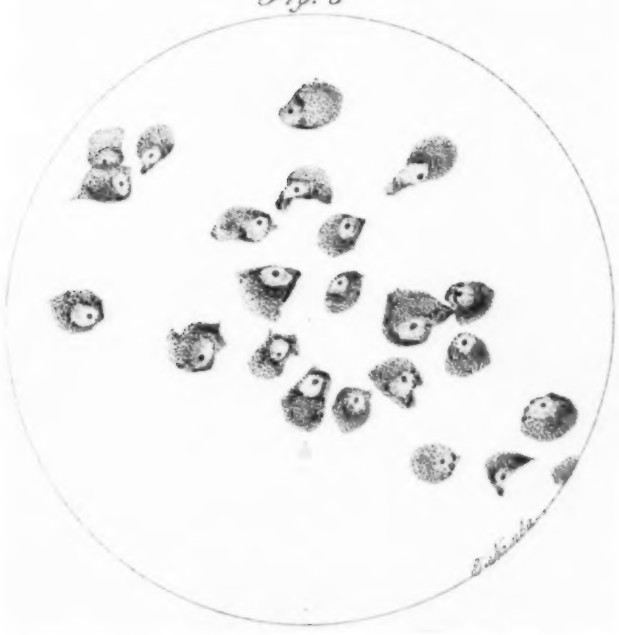

Fig. 4

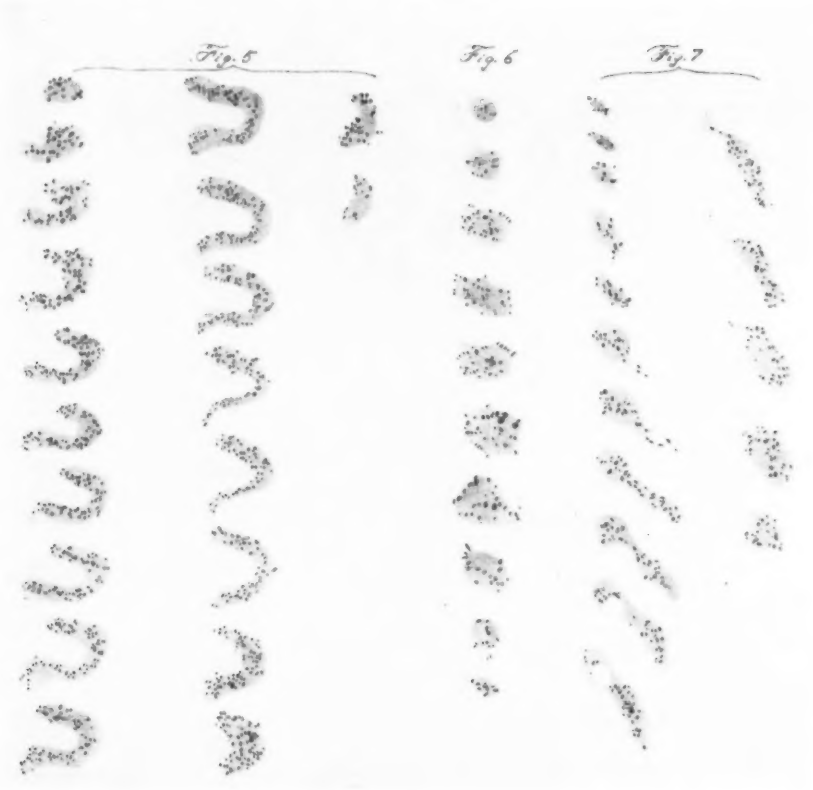
Fig. 5
Fig. 6
Fig. 7

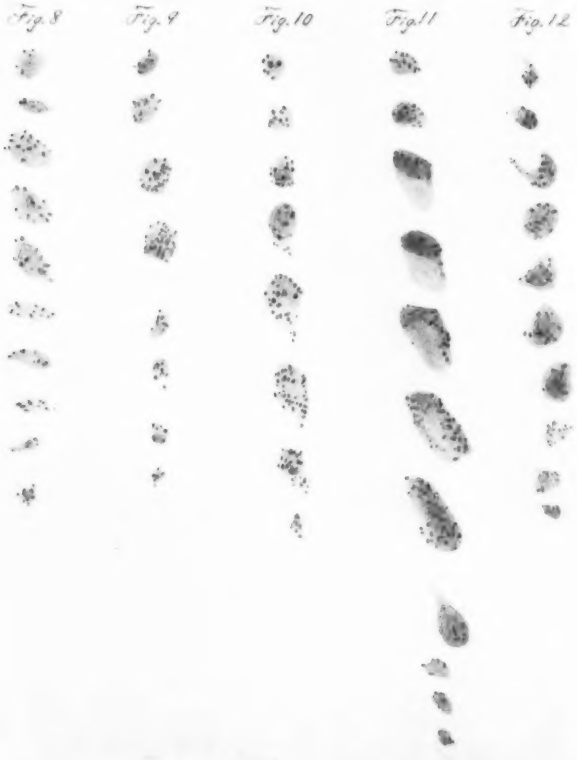
Fig. 8
Fig. 9
Fig. 10
Fig. 11
Fig. 12

sections were not well stained and others were not cut in good series.

DISCUSSION

Thirteen human brains in all were examined. The nuclei in the restiform bodies in eight of them have been described in the foregoing table. With the exception of the eighth case, which is that of a fetus 44.5 cm. long, they included brains of adults of both sexes. The remaining five cases were those of four adults and one fetus.

In all of these cases it was possible to demonstrate a peculiar nucleus, which, in transverse sections, appears in the dorso-medial part of the restiform body and is present in both embryonic and adult specimens. The inferior border of this nucleus is seen in the restiform body, about the level of the posterior vago-glosso-pharyngeal roots, and its superior border disappears just as or after the accessory auditory nucleus appears. Hence its longitudinal diameter is greater than the transverse, and averages in length about 0.5 to 1.3 mm.

The hilum of the nucleus is directed mesially, when it is well developed, as in Case I. In most cases, however, it is either absent or very indefinite, and the nucleus itself is also in an undeveloped stage, appearing in transverse sections as a small round or elliptical mass.

The following are the characteristics common to all the cases:

(1) The situation of the nucleus in the restiform body is the same in all cases.

(2) In transverse sections, when the nucleus appears elongated, the long axis runs always in a dorso-medial-ventro-lateral direction.

(3) When the nucleus appears elongated in transverse sections, it is curved and shows an incomplete hilum.

(4) The type of nerve cells in the nucleus is the same for all cases.

The type of cell found in this nucleus is similar to that of the inferior olive. The cells appear round or somewhat elliptical, and in size vary from 7.4 to 25.9 microns. The cells are uniformly scattered in the gray matter. Sometimes, however, they are more particularly collected in the periphery of the gray matter, or, more rarely, a very few nerve cells may exist outside, and not very close to, the gray matter.

The brains of certain other mammals, for instance, the monkey, dog, cat, rabbit and guinea-pig were used for similar studies, but in these thorough examination failed to reveal any such nucleus in the restiform body. Hence, it is possible that this nucleus that I have described is peculiar to the human species.

The physiology of the nucleus and its relation to the other parts of the brain are entirely unknown. It is interesting to note that the nucleus is found only in that species in which the inferior olive reaches its highest development, but not in the animals which show but a slight development of the inferior olive. This suggests that the nucleus may be a portion of the inferior olive, which has found its way into the restiform body. This hypothesis is suggested by the following findings:

(1) The nucleus presents itself in the restiform body when the inferior olive shows its greatest development.

(2) In some cases the nucleus has a hilum which bears some resemblance to a part of the inferior olive.

(3) The nerve cells of the nucleus bear a striking resemblance in shape to those of the inferior olive (Figs. 3 and 4).

(4) The restiform body and inferior olive are intimately related to each other.

(5) The fact that often only traces of the nucleus are present, and that there is a considerable amount of variation in shape and development in all cases, makes one think that the nucleus may be phylogenetically a young one, first built by man, in whom we find the greatest development of the restiform body and the inferior olive.

Although it must be freely confessed that the above, of course, is nothing but a supposition, nevertheless, judging from the morphological states of the nucleus, it is certainly a logical hypothesis.

CONCLUSIONS

(1) There is a nucleus in the restiform body of man.

(2) Tracing the transverse section from below upwards, the nucleus appears where the vago-glosso-pharyngeal root begins to disappear, and disappears where the accessory auditory nucleus begins to appear.

(3) This nucleus is found at the dorso-medial part of the restiform body on transverse sections, and extending in an elongated shape in the medulla oblongata.

(4) The nucleus has no definite shape and shows a considerable amount of variation in growth. On transverse section, the transections are different in form. Some have a hilum tending towards the medial direction, while the others present themselves as long masses extending from the dorso-medial direction to the ventro-lateral. Some of them also show the incomplete hilum-like parts in them, whereas others show only round masses.

(5) The nerve cells of the nucleus are practically identical in shape with those of the inferior olive.

(6) The nucleus is probably a portion of the inferior olive.

It is a great pleasure to acknowledge my indebtedness to Dr. K. Kosaka for his kind advice, and the excellent facilities afforded to me.

EXPLANATION OF FIGURES

FIG. 1.—Transverse section through the middle of the inferior olivary region of the human medulla oblongata (Section No. 169).

No. Inferior olive. *X, IX.* Vago-glosso-pharyngeal roots. *Crst.* Restiform body. *X.* Nucleus in restiform body.

FIG. 2.—Nucleus in the restiform body in Fig. 1, magnified.

FIG. 3.—Nerve-cells of the nucleus in the restiform body in Fig. 1 (high power).

FIG. 4.—Nerve-cells of the inferior olive in Fig. 1, that is, the part in the square (same power).

FIGS. 5-12.—Show the shape of the nuclei in the restiform bodies in Cases I to VIII, in transverse sections, arranged systematically from below upwards.

FIG. 5.—Shows the configuration of the nucleus in Case I. The nucleus is composed of nerve-cells and stroma which stain deeply with thionin. Higher up the nerve-cells increase in number, and accordingly various changes occur in the nucleus. The nucleus presents a small round outline at the beginning. Soon, however, it becomes a little bent, its concavity pointing in the dorso-medial direction, and then clearly defined dorsal and ventral spurs develop. As the size of the nucleus increases the ventral spur becomes longer than the dorsal, and its point begins to bend ventrally, so that the nucleus presents an open mouth which might be called the hilum, and appears as a succiform outline (see Figs. 1 and 2). The dorsal spur likewise begins to bend its point dorsally, both spurs finally becoming nearly the same in length and the opening gradually widening. At this stage the whole nucleus assumes the form of the letter U. Still higher up, as the hilum opens wider, the angle between both spurs becomes larger, and with the exception of a slight curve of the ventral spur, the points are no longer bent. The nucleus has gradually increased in size, and shows, in this part, its maximal cross-section, its hilum pointing towards the median raphé. Again, higher up, when the inferior portion of the accessory auditory nucleus appears, the nucleus begins to decrease in size, and shortly afterwards, assuming its original form, it disappears. The nerve-cells of the nucleus, for the most part, tend to become round, but in some instances they are somewhat fusiform (Fig. 3), and approximately of the same size, varying from 11.1 to 22.2 microns. The longitudinal diameter (the distance between the inferior and superior ends) of the nucleus measures about 1.3 mm.

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The Croonian Lectures on the Psychology of the Special Senses and Their Functional Disorders. Delivered before the Royal College of Physicians in June, 1920. By Arthur F. Hurst, M. A., M. D. (Oxon.), F. R. C. P. 1920. 8°. 123 pages.

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Textbook of Nursing Procedures By Anna C. Jammé, R. N. 1921. 12°. 140 pages. Macmillan Company, New York.

PUBLICATIONS

The following twelve monographs:

Benzol as a Leucotoxin. By LAURENCE SELLING, M. D. 60 pages. Price, $1.00.

Primary Carcinoma of the Liver. By M. C. WINTERNITZ, M. D. 42 pages. Price, 75 cents.

The Statistical Experience Data of The Johns Hopkins Hospital, Baltimore, Md., 1892-1911. By FREDERICK L. HOFFMAN, LL.D., F.S.S. 161 pages. Price, $2.00.

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Leukaemia of the Fowl: Spontaneous and Experimental. By HARRY C. SCHMEISSER, M. D. Price, $2.00.

The Structure of the Normal Fibers of Purkinje in the Adult Human Heart and Their Pathological Alteration in Syphilitic Myocarditis. By O. VAN DER STRICHT and T. WINGATE TODD. Price, $2.00.

The Operative Story of Goitre. The Author's Operation. By WILLIAM S. HALSTED, M. D. Price, $3.50.

Study of Arterio-Venous Fistula with an Analysis of 447 Cases. By CURLE L. CALLANDER, M. D. Price, $2.50.

Ligations of the Left Subclavian Artery in its First Portion. By WILLIAM S. HALSTED. Price, $2.00.

The Pathology of the Pneumonia in the United States Army Camps During the Winter of 1917-18. By WILLIAM G. MACCALLUM. Price, $1.50.

Pathological Anatomy of Pneumonia Associated with Influenza. By WILLIAM G. MACCALLUM. Price, $2.00.

are now on sale by THE JOHNS HOPKINS PRESS, Baltimore. Other monographs will appear from time to time.

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(Foreign postage, 50 cents.) Price of cloth-bound volumes, $5.00 each.

A complete index to Vols. I-XVI of the Bulletin has been issued. Price 50 cents, bound in cloth.

NOTES ON NEW BOOKS

Injuries and Diseases of the Bones and Joints. By FREDERICK H. BAETJER and CHARLES A. WATERS. Cloth, $10.00. (*New York, Paul B. Hoeber, 1921.*)

This volume is in many ways distinctive. It is a contribution of the highest order—one in which quality is the keynote throughout—a type which we always look for but seldom find. The usual stereotyped and space-consuming features of most books are omitted. Technique, statistics, theories and the historical side of roentgenology are not considered. Nowhere is there the slightest taint of padding either in writing or illustrations.

It is a purely clinical contribution, dealing only with facts proven beyond question by correlation of the X-ray shadows with the actual anatomical and pathological findings at operation and necropsy. Few roentgenologists, if any, are more qualified by experience, study, and keenness of observation to pass judgment upon the clinical import of roentgenology. It is a big story (covering the entire roentgenological output of The Johns Hopkins Hospital) remarkably condensed and told in a delightfully simple and direct way.

The outstanding features of the work are its thoroughly practical character, and the intimate and accurate views on the problems of medicine and surgery and of the morbid anatomy in each field of study. Fractures, dislocations, joint lesions, bone infections, bone tumors, various abnormalities and dystrophies are considered from every angle of diagnosis. The book is so well balanced that unusual features are not conspicuous. The chapter on bone tumors, however, is particularly impressive. This very complicated and poorly understood subject is reduced to unusual simplicity by the writers' consummate knowledge of the subject from the clinical, pathological and roentgenological aspects.

The book represents the very latest and the most advanced information on the interpretative side of roentgenology. It should be indispensable to all concerned with clinical problems. The illustrations are well selected and clearly reproduced. There is only one regret—that a subject so admirably presented and of such importance should include only a part of clinical roentgenology. It is to be hoped the remainder of the field will shortly be covered by a supplement to this volume.

W. E. D.

Dermatology, The Essentials of Cutaneous Medicine. By WALTER J. HIGHMAN. (*New York, MacMillan Co., 1921.*)

To the reviewer the appearance at this time of still another text-book on diseases of the skin naturally awakens a sense of critical suspicion. He fortifies himself, however, in the thought that perhaps the author of such a book will bring something new to a field of medicine already progressing vigorously to the point at which respectful attention is being given it. Highman, in his initial effort, has certainly allayed any suspicion of giving us the usual matter-of-fact presentation of the subject.

In the preface, splendidly set forth as it is, he strikes at once at the sore spots which affect this special field, so far as teaching and exposition are concerned. Thus, when he states that the true dermatologist is "an internist who knows the skin" he is stating at once the vital basis on which modern dermatology rests and from which it should be taught. He undoubtedly attempts, thus, to bring back the specialty to the field from which, since 1860, it has strayed perforce through over-enthusiasm and through the unfortunate idea that all skin diseases were of external origin. The schools of Hebra and of Kaposi made such a great stride forward that they left too much behind.

The book is intended for students—being the essentials of cutaneous medicine—but it must certainly appeal also to the busy doctor who wishes to orient himself, briefly, regarding the newer conceptions of the dermatoses. It has a peculiar flavor of originality in so far as an honest attempt is made to clarify our ideas more especially regarding etiology. The author has, of necessity, been compelled to follow in the main a classification of the dermatoses similar to that of the modern treatises; but wherever possible, he has taken the step forward which gives us a saner idea of the causes of the common diseases of the skin. It is, of course, beyond the scope of this review to enumerate this classification.

Despite the necessary brevity of the discussions, Highman has not hesitated to give a relatively protracted exposition of certain diseases. For example, the chapter on urticaria and on the vesicular diseases of the skin certainly makes an eloquent appeal to the critical mind and one must not fail to appreciate that the student in particular needs at times just such a dissertation on a subject so fraught with confusing ideas. The latter chapter (vesicular diseases) is worth alone the writing of the book; for it gives us the most appealing and sensible account of the hitherto confused conceptions of the relationship of the eczemas and the dermatitides. This much should be said in favor of Highman's sensible attitude regarding this point: that even if the passing of the years may prove him to be in error, he has, at least, given the reader and the student a tentative working basis for a simpler and more acceptable comprehension of the one subject which has hitherto made dermatology difficult of understanding, unsatisfying in teaching, and embarrassing in explanation to both the tyro and to the trained worker. Finally, the chapters on syphilis are adequate so far as the size of the book is concerned, very pithy, and certainly quite up to date.

The book, however, is not without some minor faults. The author's experiences in treatment, while much to be commended, are sometimes decidedly debatable, from the standpoint of those of others. In this connection the use of arsenic in psoriasis might be mentioned. In the main, however, his ideas on therapy are excellent and should be helpful—they are brief, practical, and not over-worked; and his prescriptions, happily, are given in the metric system.

He does not mention the possibility of recurrence of the eruption in pityriasis rosea; and unless we mistake, he does not include a description of pellagra.

It is questionable whether the almost complete omission of a brief histological description of the important dermatoses is a wise thing; and further, whether the omission of a brief bibliography has any decided advantage. If the author of a procedure or of a contribution is worth mentioning, it appears to us that some reference to the source would be worth while and would not take up much useful space. Finally, there is fairly frequent evidence of faulty typography—a fault very readily corrected, of course; and there is evidence also of some carelessness in the use of words.

It should be stated, moreover, that the illustrations which number nearly a hundred are of a type of selection and of an excellence which has no peer in any other text-book with which we are acquainted. The volume is of handy size, clearly printed and attractively arranged. And best of all, the author has a literary style which is characterized by such individual ease and clarity of expression, by such original phrasing that one experiences a genuine pleasure in reading the book. Such factors are bound to give a book originality; and having this quality it should certainly win a place as a real contribution to dermatological teaching.

I. R. P.

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